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EFFECT OF A HYPOCRETIN/OREXIN ANTAGONIST ON NEUROCOGNITIVE PERFORMANCE

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14. ABSTRACT
During Year 5, we continued tests of the hypothesis that disfacilitation of wake-promoting systems by the hypocretin (Hcrt) receptor antagonist almorexant (ALM) results in less functional impairment than the inhibition of neural activity produced by the benzodiazepine receptor agonist zolpidem (ZOL). One paper was published (Morairty et al. 2014), another has been accepted for publication (Dittrich et al., in press) and a third is in resubmission (Vazquez-DeRose et al., submitted). Data collection for Aims 2c and 3b.2 have been completed and data analysis ongoing; manuscripts will be written and submitted during Year 6. Data collection and analysis of Aim 3a is nearing completion; an abstract summarizing this work has been submitted for presentation at the 2014 Society for Neuroscience meeting. Data collection for Aims 3b.3, 4c and 6a have been initiated. The overall results obtained to date are consistent with the hypothesis that the hypocretin/orexin antagonist ALM produces less functional impairment than the benzodiazepine receptor agonist zolpidem (ZOL) because ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems.

15. SUBJECT TERMS
Sleep, performance, drug, neurotransmitter, hypocretin, orexin, benzodiazepine, zolpidem, neurochemistry, microdialysis

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PROGRESS REPORT

"Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance"

USAMRAA Grant W81XWH-09-2-0081

DR080789P1

Year 5: 8/1/13 to 7/31/14

Thomas S. Kilduff, Ph.D., Principal Investigator

INTRODUCTION

Almorexant (ALM) is a hypocretin/orexin (Hcrt) receptor antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Preclinical data demonstrate that animals treated with ALM are easily aroused from sleep and are free of ataxia and other behavioral impairments. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. The overall hypothesis that underlies this research is that ALM produces less functional impairment than the benzodiazepine receptor agonist zolpidem (ZOL) because ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems. Whereas the human study component (W81XWH-09-2-0080; Thomas Neylan, M.D., Principal Investigator) will establish whether ALM is superior to ZOL in neurocognitive tests, the animal studies (W81XWH-09-2-0081; Thomas Kilduff, Ph.D., Principal Investigator) will compare the neural circuitry that underlies the activity of these compounds, their effects on sleep and performance, and the effects of these compounds on biomarkers associated with normal sleep.

BODY

Task 2. *Test the hypothesis that rodents receiving ZOL will show greater neurocognitive impairment than those receiving ALM or PBO.*

2a. Assessment of Almorexant effects on spatial reference memory in rats.
Status: Data collection and analysis COMPLETED; paper published in January, 2014.

2b. Assessment of Almorexant effects on spatial working memory in rats:
Status: Data collection and analysis COMPLETED; paper published in January, 2014.

2c. Assessment of Almorexant effects on psychomotor vigilance in rats
Status: Data collection completed; analysis ongoing (see below).

2d. Synthesis of ALM (months 1-4).
Status: COMPLETED

Progress – Task 2a and 2b: Tasks 2a and 2b have been completed and an article entitled "The hypocretin/orexin antagonist almorexant promotes sleep without impairment of performance in rats" was published in *Frontiers in Neuroscience* in January, 2014.

Progress – Task 2c: The studies assessing the effects of ALM in the rodent psychomotor vigilance (rPVT) have been completed. Our results are described below.

Methods: The general protocol for the rPVT is as follows (**Figure 1**). Rats were motivated to perform the operant rPVT task for water reinforcements by having water unavailable to them for 23 h prior to all operant training and testing. Rats were gradually acclimated to the water restriction schedule over several days by reducing the amount of time

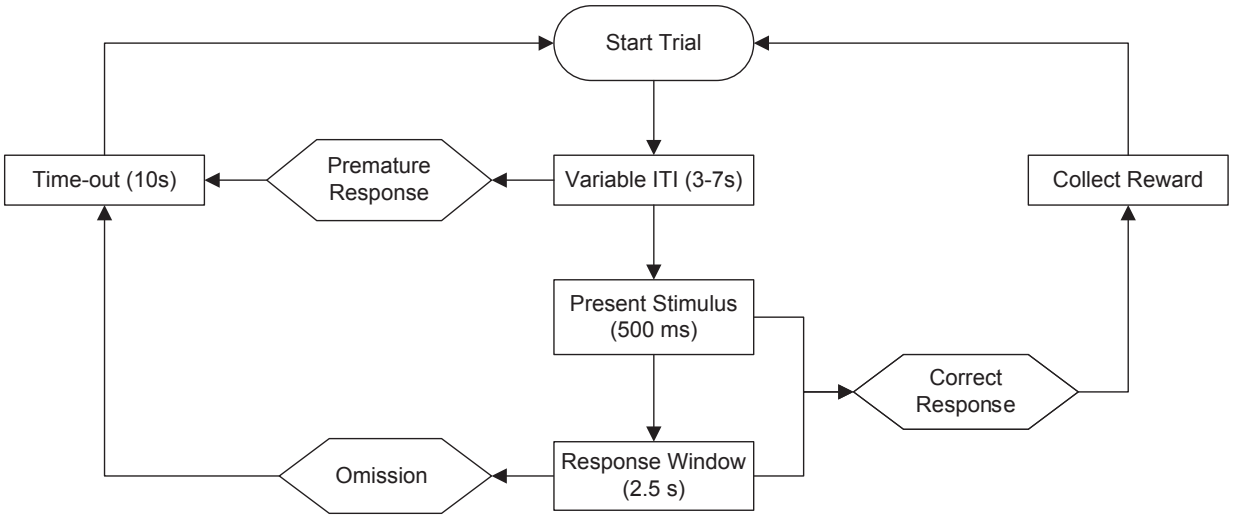

Figure 1. Flowchart of the rodent Psychomotor Vigilance Task. During the task, the inter-trial interval (ITI) varied from 3 to 7 s in 1 s increments in a quasi-random fashion (equal density of intervals throughout session). Responses during the ITI (a 'premature response') or failing to respond within 3 s of the stimulus presentation (an omission) were treated as errors and resulted in a 10 s 'time-out' (housing light extinguished and absence of trials). At the end of the 10 s time-out, the house light was re-illuminated and a new ITI started.

each day that water was available in the home cage. rPVT training took 3 mo to complete. Following this 3 mo training period, rats that did not meet criteria (> 100 correct responses per test session) were removed from the study. rPVT testing consisted of a stimulus light on for a duration of 0.5 s followed by a 3 s response period. The intertrial interval varied between 3-7 s. Errors resulted in a 10 s "time out" period during which the dim house lights were turned off. Test measures were the following:

- Correct responses (**CR**): Responding during stimulus presentation or within the response window.
- Omission (**OM**): Failure to respond within the 3 s window of opportunity.
- Premature errors (**PE**): Responding during the inter-trial interval.
- Response latencies (**RL**): Time from stimulus onset to a correct response.
- Numbers of trials: Total number of trials per session.
- Number of responses: Number of entries in the reward trough (data not shown).
- Lapses: Trials in which response latencies were >2x the average basal response latency for each rat.

Results: Seventeen rats were implanted with telemetry for devices for EEG recordings. Of these, 4 rats did not meet criteria following 3 mo of training and were removed from the study. We anticipated that up to a third of the rats might not meet criteria, so these results were expected. In addition, 2 rats had transmitter malfunctions prior to completion of the study and could not be included in our results. Therefore, 11 rats completed the rPVT study.

When the testing was about to begin, rats were acclimated to the dosing procedure by administration of 1 ml of VEH (p.o.). However, when we examined the performance following this dose of VEH, we found a significant decline in all rPVT measure. Therefore, we reformulated the VEH solution using a base of physiological saline rather than just H_2O. This

reformulation was effective at keeping the rats' performance in the rPVT above minimum criteria following dosing with VEH.

When the experiments were initiated, it became clear very early on that there were significant deficits in performance following ZOL at 100 mg/kg, p.o. Some rats had very few responses to the stimulus following ZOL. In addition, ALM-treated rats showed a noticeable deficit compared to VEH. Therefore, we added 2 additional conditions, ALM and ZOL at 30 mg/kg (p.o.). These additional concentrations of ALM and ZOL have been shown to be sleep-promoting but at more moderate levels compared to 100 mg/kg doses.

While performance in the rPVT declined following ALM and ZOL at both concentrations, the magnitude of the decline was significantly greater following ZOL (**Figure 2**). All rPVT performance measures decreased significantly following ZOL administration. Following ZOL, CR and the number of trials decreased while OM, response latencies and lapses increased. Interestingly, the number of PE decreased; following ZOL, rats were simply engaging less in the task. While ALM showed a decrease in sustained attention (decreased CR and the number of trials, increased OM and lapses), no impairment was seen in RL or PE. Further, the effects on CR, OM, the number of trials and lapses were greater following ZOL than ALM.

To investigate deeper into rPVT performance, we determined the density distributions for response latencies following all conditions (**Figure 3**). For both VEH and ALM, most responses occurred in less than 0.5 s. and the density distribution patterns of the VEH and ALM were similar. Following ZOL, however, the density distribution showed a much broader distribution across the response period. These data show that rats performed equally as well following ALM as following VEH, while responses often occurred more slowly in the presence of ZOL.

Changes in rPVT performance could not be attributed to prior sleep history. As can be seen in **Figure 4**, rats slept equivalent amounts for the hour prior to testing following ALM and ZOL. However, while the EEG power spectra during NREM sleep following VEH and ALM were indistinguishable (**Figure 5**), ZOL was followed by very large changes across the entire NREM EEG power spectrum (**Figure 6**). While the full meaning of such changes in the EEG power spectrum is yet to be understood, these data support the hypothesis that ALM produces physiological sleep while ZOL produces generalized CNS inhibition that results in a pharmacological, rather than physiological, sleep state.

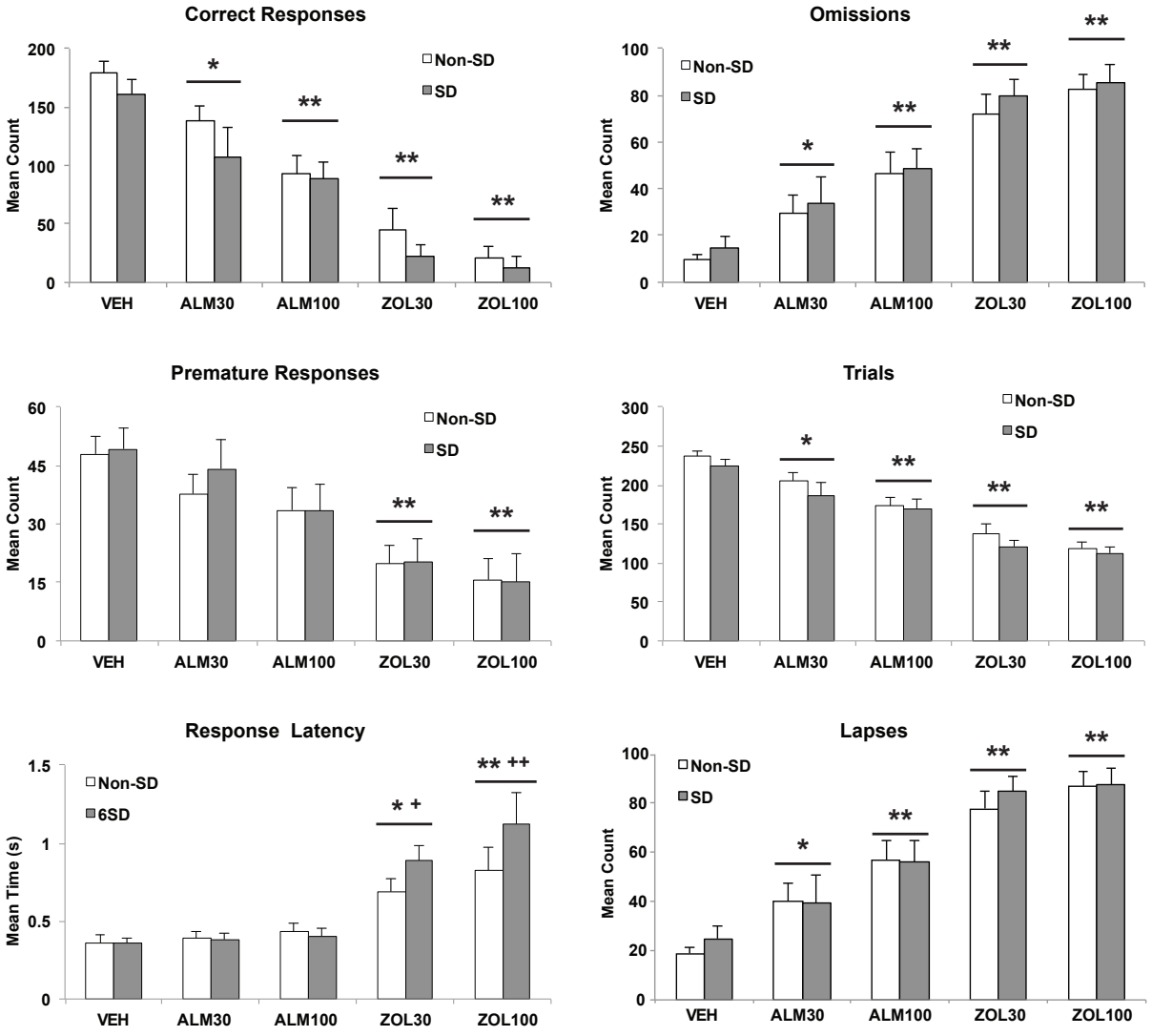

Figure 2. rPVT outcome measures. rPVT performance decreased significantly across all measures following ZOL administration. While ALM showed a decrease in sustained attention (decreased CR, increased OM and lapses), no impairment was seen in RL. Data shown as group mean ± SEM (n = 9–10). Multiple comparisons vs. control group (Bonferroni t-test): *= $p < 0.05$ significantly different from vehicle condition. ** = $p < 0.01$ significantly different from vehicle condition. + = $p < 0.05$ significantly different SD condition within drug treatment. ++ = $p < 0.01$ significantly different SD condition within drug treatment.

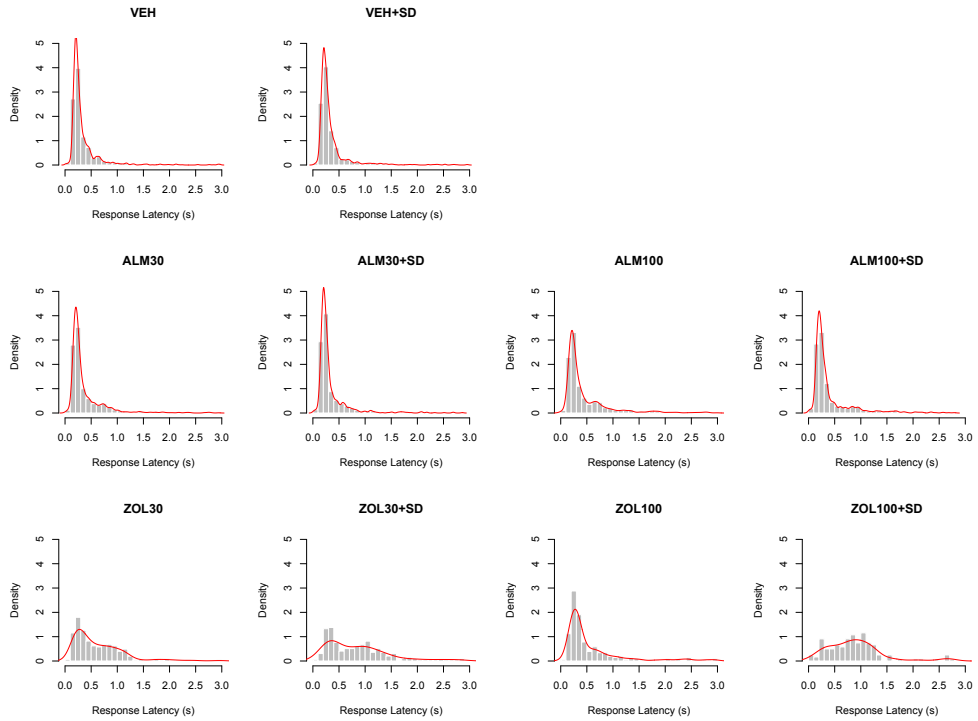

Figure 3. Density distributions for the response latencies in each test condition. RL distributions are similar for VEH and Almorexant (ALM) following either baseline or SD conditions. However, ZOL administration shifted the RL distributions following both baseline and SD conditions.

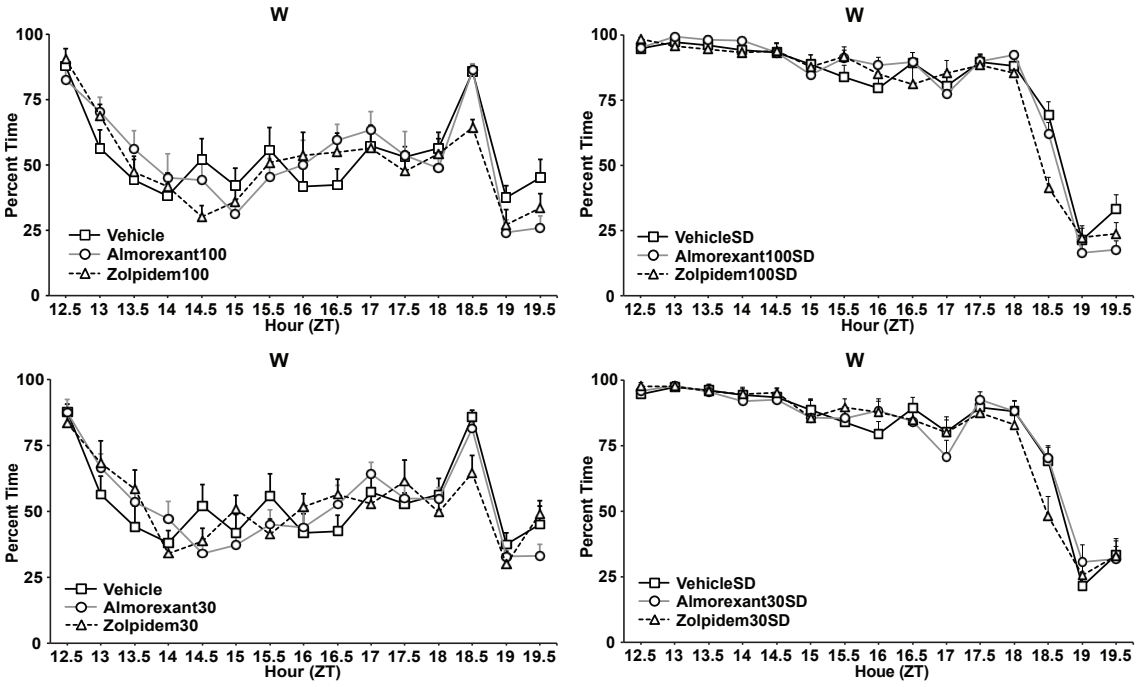

Figure 4. Effects of Almorexant and Zolpidem (each at 100 mg/kg and at 30 mg/kg po) on time spent awake under baseline conditions (left panel) or following 6 h of SD (right panel) during the active phase (lights off). Note that for the 60 min prior to rPVT test, the doses of Almorexant and Zolpidem were equally effective at inducing sleep.

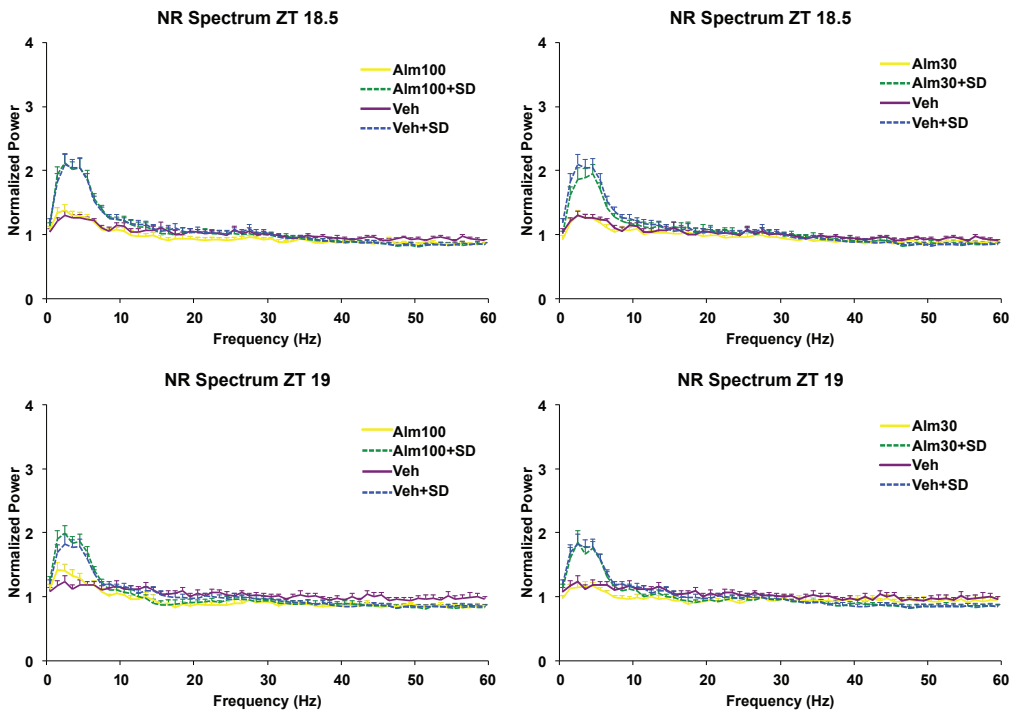

Figure 5. Rats showed no significant differences in EEG spectra during NREM sleep at 30 min (top) or 60 min (bottom) following administration of Almorexant (Alm) when compared to vehicle (Veh).

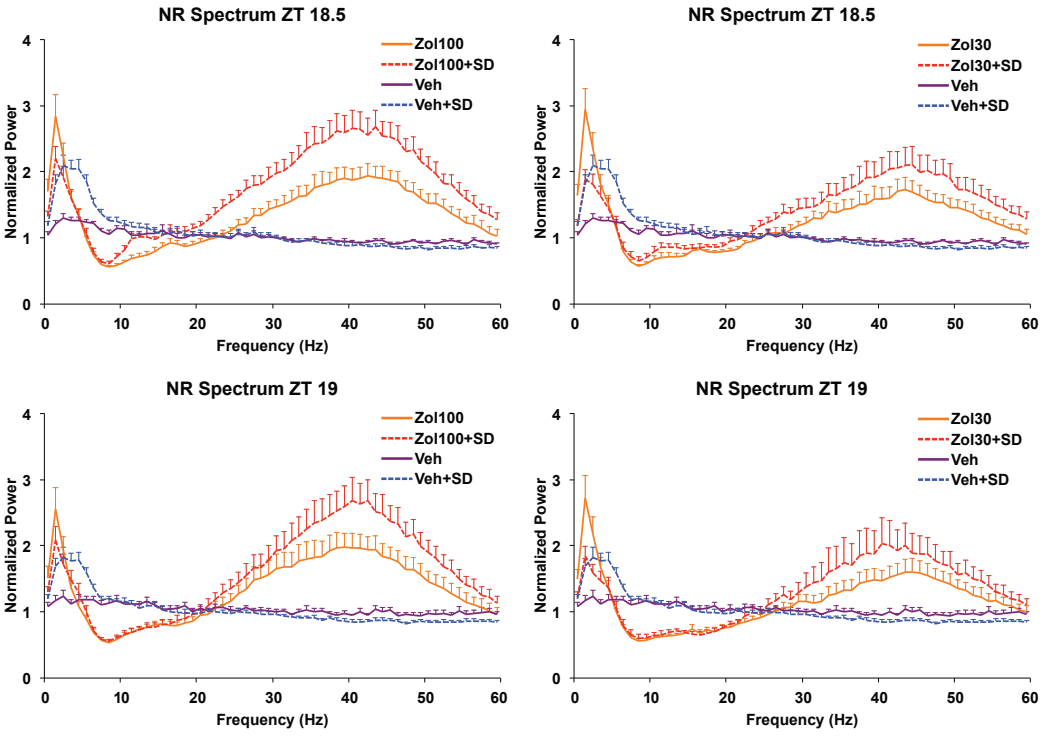

Figure 6. Rats showed significant alterations in EEG spectra during NREM sleep (0-60 Hz at 1 Hz resolution) 30 min (top) and 60 min (bottom) after administration of Zolpidem (Zol; left panel: 100 mg/kg; right panel: 30mg/kg) compared to vehicle (Veh). These changes appear to be dose-dependent and are further potentiated following 6 h of sleep deprivation (SD) during the active phase.

Task 3. *Test the hypothesis that the Hcrt antagonist ALM induces sleep by selectively disfacilitating the activity of the histaminergic, serotonergic, noradrenergic and cholinergic wake-promoting systems whereas the BzRA ZOL causes a generalized inhibition of the brain.*
3a. Double-label immunohistochemistry with Fos and phenotypic markers.
Status: Data collection and analysis ongoing; see below.

3b. Assessment of hypnotic efficacy in saporin-lesioned rats.
3b.1 Status: Basal forebrain lesion study COMPLETED; manuscript in revision for publication in *Brain Structure and Function*.
3b.2 Status: Locus coeruleus lesion study COMPLETED; manuscript to be written.
3b.3 Status: Tuberomammillary nucleus lesion study ongoing; see below.

Progress – Task 3a: We proposed to determine whether ALM and / or ZOL disrupt activation of several wake-promoting neuronal populations during forced wakefulness and undisturbed conditions using Fos as a marker for neuronal activation. Task 3a is nearly complete as all data collection and most histological stains have been completed, but some analysis remains. Completion of Task 3a was delayed because data needed to be collected from 12 additional rats this year, bringing the total utilized to N=88 for this study.

Methods: Rats were given 1 mL (p.o.) ALM (100mg/kg), ZOL (100mg/kg), or VEH at their mid-active phase (ZT18). Half of the animals were left undisturbed for 1.5h after dosing, while the other half of the rats were sleep deprived (SD) by gentle handling for 1.5h. Animals were then deeply anesthetized and perfused, and the brains were removed and sectioned on a freezing microtome. Double label immunohistochemistry for Fos and markers for wake-active neurons (histamine (HA), hypocretin (Hcrt), serotonin (5-HT), and acetylcholine (ACh)) was performed using coronal sections of tissue from the appropriate brain region. Additionally, the number of single labeled Fos-positive nuclei in the locus coeruleus (LC) was quantified.

Results: In HA neurons, both VEH- and ALM-treated rats exhibited significantly greater Fos coexpression with adenosine deaminase (ADA) following SD than did ZOL-treated animals (**Figure 7**). This result indicates that activation of HA neurons is unimpaired by ALM whereas ZOL inhibits such activation.

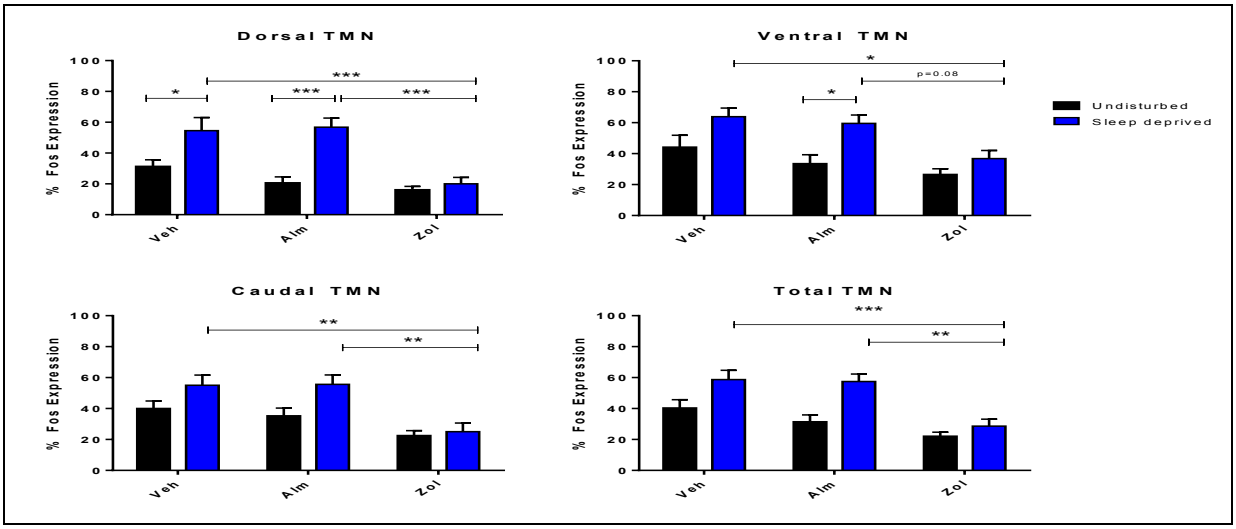

Figure 7. Effect of drug treatment on Fos expression of wake-active HA neurons in the dorsal, ventral and caudal subdivisions of the tuberomammillary nucleus (TMN) of the hypothalamus. Black bars depict group means for undisturbed rats, blue bars for SD rats. *, **, *** = p<.05, 0.01, and 0.001, respectively.

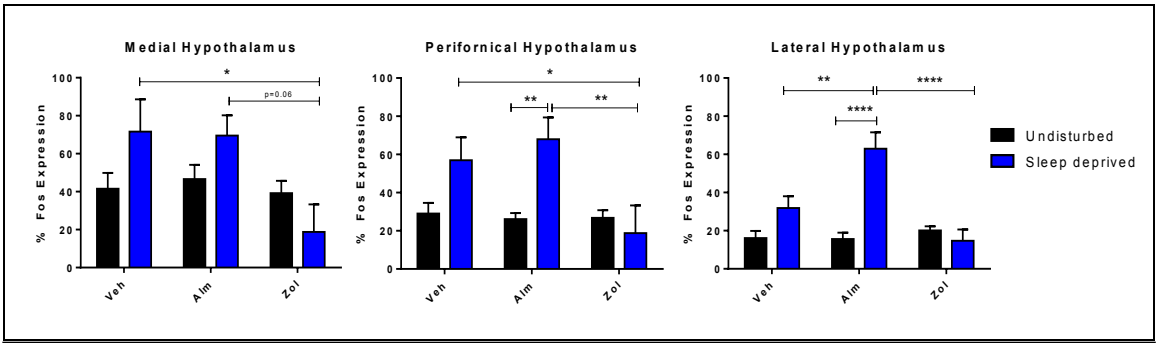

Figure 8. Effect of drug treatment on Fos expression in hypocretin neurons found in three hypothalamic regions. Black bars depict group means for undisturbed rats, blue bars for SD rats. *, **, *** = p<.05, 0.01, and 0.001, respectively.

We also conducted additional processing and analysis of Hcrt double-labeling experiments. The results of this analysis confirm the preliminary results reported last year indicating that a greater proportion of Hcrt neurons express Fos in VEH- and ALM-treated animals after SD than in ZOL animals (**Figure 8).** This indicates that ALM does not impair SD-induced activation of Hcrt neurons, whereas ZOL inhibits activation of this neuronal group.

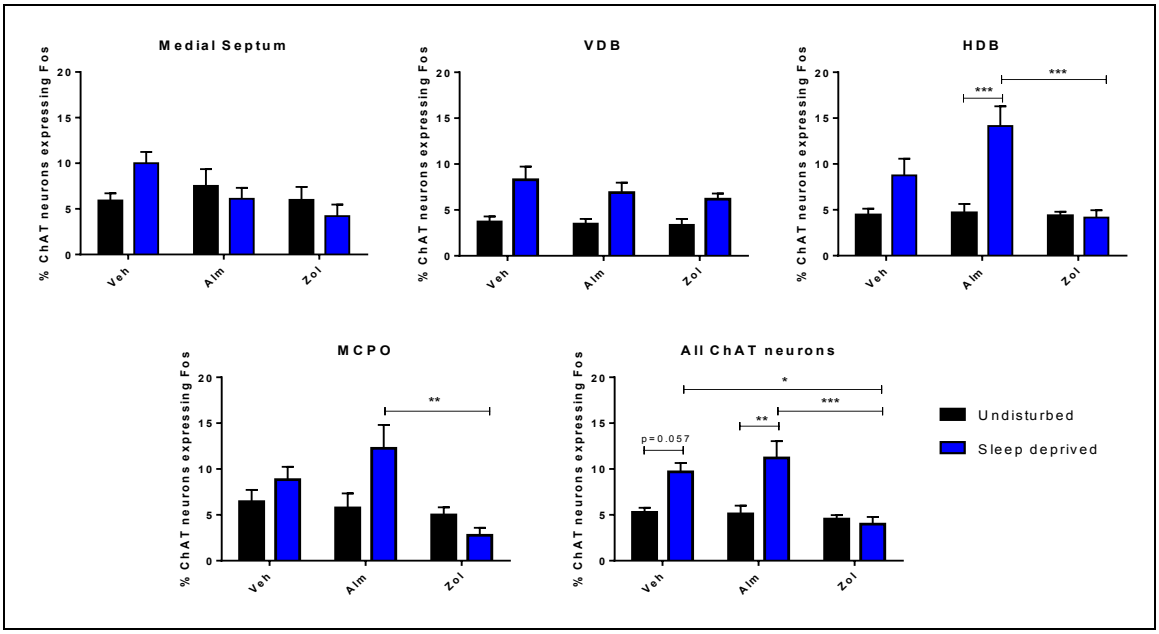

Figure 9. Effect of drug treatment on Fos expression in wake-active cholinergic neurons in four regions of the basal forebrain. Black bars depict group means for undisturbed rats, blue bars for SD rats. *, **, *** = p<.05, 0.01, and 0.001, respectively.

To investigate whether wake-active Ach neurons in the basal forebrain are affected by ALM and ZOL, neurons that express choline acetyltransferase (ChAT), a marker for ACh, were scored for Fos coexpression. In the horizontal diagonal band of Broca (HDB) and magnocellular preoptic nuclei (MCPO), ALM-treated animals expressed significantly greater Fos in ACh neurons than did ZOL-treated animals following SD (**Figure 9**). When ACh neuron counts

across all subregions were consolidated, both VEH- and ALM-treated animals were found to express Fos in a significantly greater proportion of Ach neurons than did ZOL-treated animals. These results indicate that, unlike ZOL, ALM does not inhibit activation of Ach neurons.

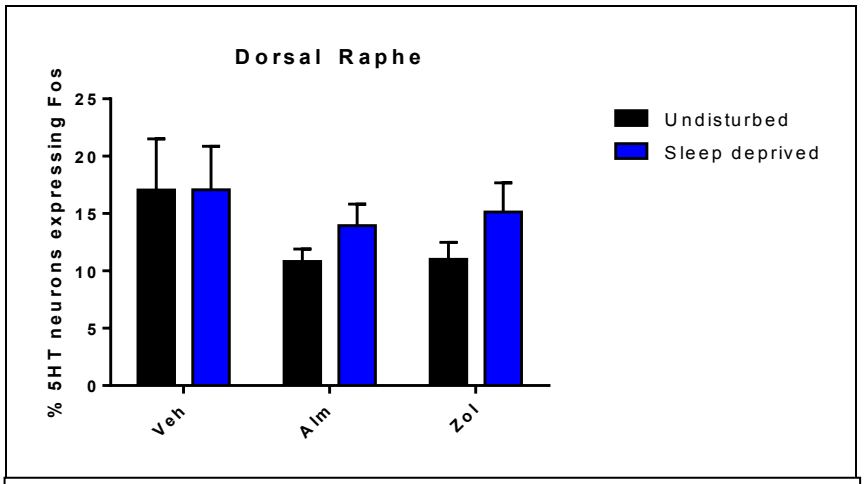

Figure 10. Effect of drug treatment on Fos expression in wake-active 5-HT neurons in the dorsal raphe nucleus. Black bars depict group means for undisturbed rats, blue bars for SD rats.

5-HT expressing neurons in the dorsal raphe nucleus were scored for Fos double labeling. No significant changes in Fos expression were observed between VEH-, ALM-, or ZOL-treated rats either under SD or undisturbed conditions (**Figure 10**), indicating that neither ALM or ZOL affect Fos expression in 5-HT neurons.

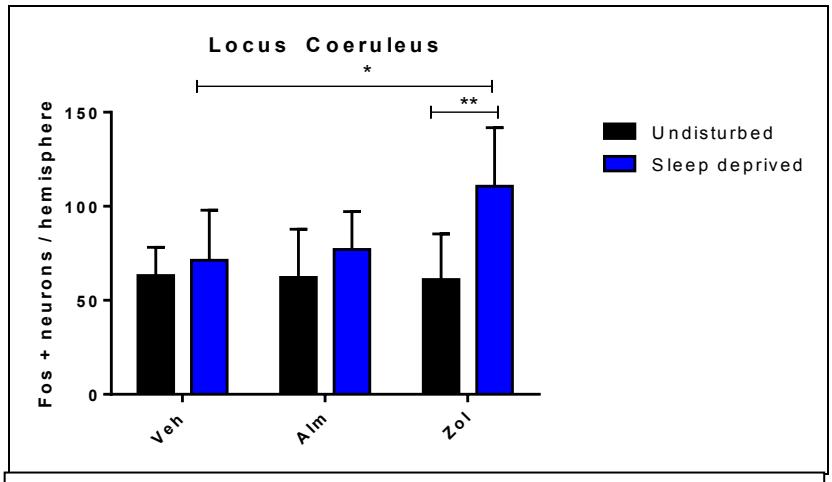

Figure 11. Effect of drug treatment on Fos expression in the locus coeruleus (LC). Black bars depict group means for undisturbed rats, blue bars for SD rats.

The total number of Fos-positive nuclei in a selected region of the locus coeruleus (LC) was counted in order to assess the effect of ALM and ZOL on LC activation. We found that SD did not affect the number of Fos positive nuclei for VEH- or ALM-treated rats, but that ZOL-treated rats exhibited significantly greater Fos expression in this region following SD (**Figure**

11). This is likely a consequence of the increased level of handling required to keep ZOL-treated animals awake during the SD protocol compared to VEH- or ALM-treated rats.

Progress – Task 3b: As described in previous Progress Reports, we have completed two of three lesion studies testing the hypothesis that ALM induces sleep by selectively disfacilitating the activity of subcortical wake-promoting systems. Rats with bilateral lesions of the basal forebrain (BFx) exhibited a decrease in ALM-induced NREM sleep compared to sham-operated rats (Shams), whereas ZOL showed full efficacy in promoting NREM sleep in BFx and Sham rats. The manuscript describing these results is in revision for publication in *Brain Structure and Function*.

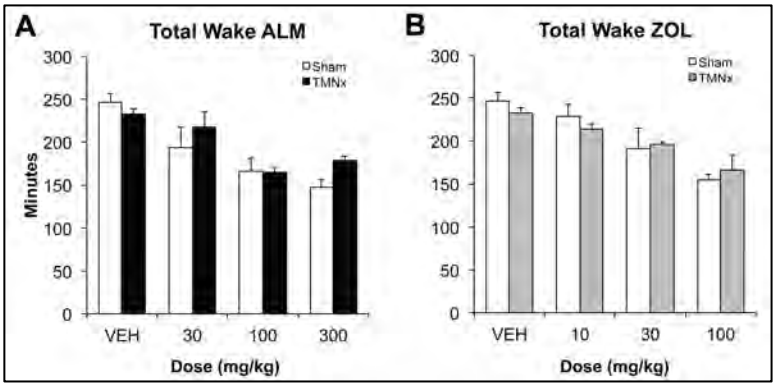

Figure 12. Cumulative wake time for 6 h after dosing with ALM (**A**), ZOL (**B**) or Veh at lights-out (ZT12). Vehicle doses are presented in both graphs. N=2 TMNx, 2 Sham.

Locus coeruleus lesions (LCx) attenuated ALM-induced but not ZOL-induced decreases in NREM sleep latency, and attenuated ALM-induced increases, but not ZOL-induced decreases, in REM sleep compared to Shams. The manuscript describing these results is currently being written.

This year, we initiated the third lesion study and assessed the response to ALM and ZOL following lesions of the histaminergic tuberomammillary neurons (TMNx) of the posterior hypothalamus.

Methods: Adult male rats were injected bilaterally with 250-300 nL of the neurotoxin saporin conjugated to Hcrt2 (Hcrt-SAP; 228ng/μL) using a Hamilton syringe connected to a digital microinjection pump at -4.2mm AP, ± 0.8mm ML from bregma, and -9.3mm from dura. Rats were instrumented for EEG at this time, and following full recovery were administered HPMC vehicle, ALM (30/100/300 mg/kg) or ZOL (10/30/100 mg/kg) p.o. in fully balanced order at lights-out. Sleep EEG was scored for the first 6 h following dosing.

Results: Despite positive results in placing this lesion in pilot studies, only 2 of 8 experimental rats exhibited complete bilateral TMN lesions. Statistical evaluation was precluded due to the low sample size, but TMNx appeared to attenuate the efficacy of ALM 300 mg/kg at reducing wakefulness (**Figure 12A**). By contrast, ZOL exerted more similar reductions in wakefulness in TMNx and Sham rats (**Figure 12B**). While additional rats will be needed to complete the study, these preliminary results are encouraging.

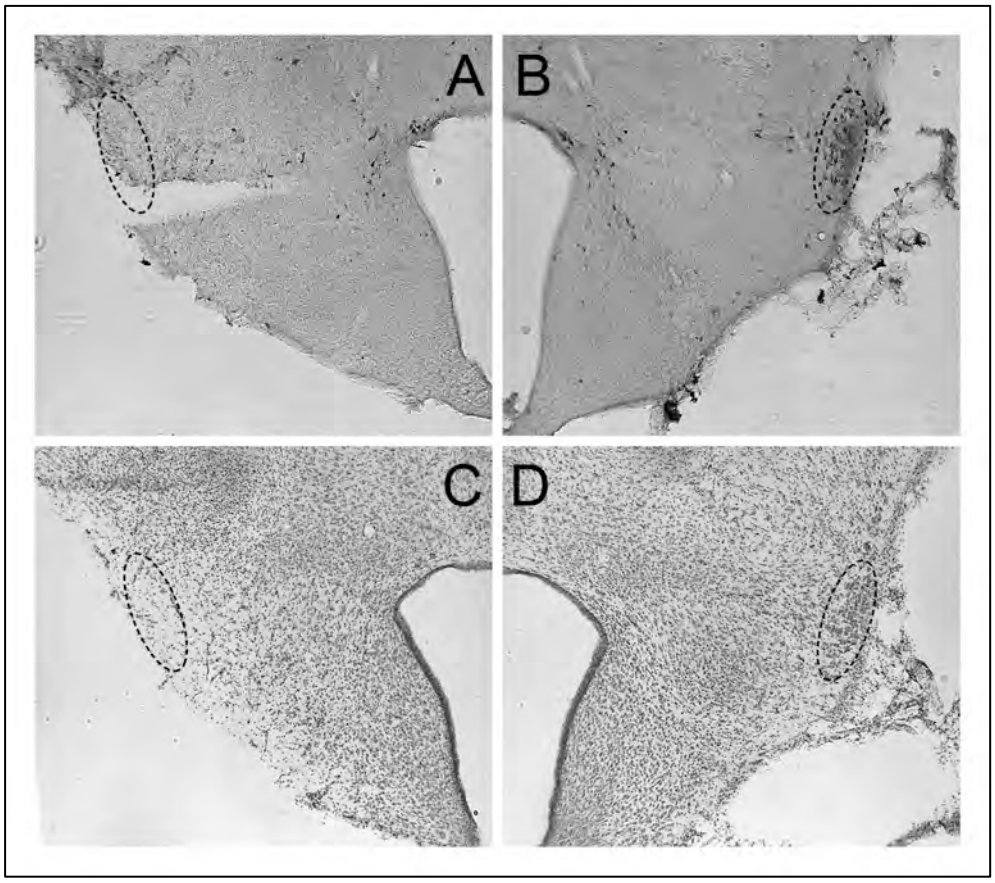

Figure 13. Representative unilateral TMN lesion from a rat injected with 360nL of HCRT-SAP in the left TMN (Panels A &C). Brain sections were immunostained for adenosine deaminase (A-B), with adjacent sections stained for Nissl (C-D). Dotted ovals indicate the location of the TMN; Histamine-positive neurons are evident in the right TMN (B) but not the left (A).

In a second round of pilot studies we refined the lesion placement technique. 8 rats were injected unilaterally with 360nl (N=4) or 540nL (N=4) of Hcrt-SAP (228ng/μL) using calibrated pulled glass micropipettes and a Picospritzer at either 4.2mm (N=4) or 4.35mm (N=4) posterior to bregma (ML and DV coordinates were -0.8mm from bregma and -9.3mm from dura, respectively) and perfused 2 weeks later. 4 of 4 rats injected at -4.35mm AP exhibited a TMN lesion ipsilateral to the injection site (**Figure 13A, C**), as indicated by immunostaining for ADA (**Figure 13A, B**) and by Nissl stain (**Figure 13C, D**). By contrast, only 1 of 4 rats injected at -4.2mm had a lesion. This posterior adjustment of injection coordinates is thus likely to substantially improve injection accuracy.

Task 4. *Test the hypothesis that ALM, but not ZOL, induces sleep by facilitating the mechanisms that underlie the transition to normal sleep.*

4a. Effects of ALM and ZOL on sleep-active brain areas.
Status: Data collection and analysis COMPLETED; manuscript in press.

4b. BF adenosine (ADO) release in response to oral ALM and ZOL.
Status: Data collection and analysis COMPLETED; manuscript in review.

4c.2 Effects of BF microinjections of ALM and ZOL on sleep/wake and neurotransmitter release in the cerebral cortex.
Status: See report below.

Progress - Task 4a: This study was described in detail in last year's Progress Report. We can now report the manuscript entitled "Homeostatic Sleep Pressure is the Primary Factor for Activation of Cortical nNOS/NK1 Neurons" was accepted for publication in the journal *Neuropsychopharmacology* on 17 July 2014.

Progress - Task 4b: Data collection and analyses for Tasks 4b and 4b.2 were completed by end of September, 2013. To form a complete story, we additionally added a subset of basal forebrain behavioral and pharmacological data from Task 3 for our manuscript. The manuscript entitled, "Hypocretin/orexin Antagonism Enhances Sleep-related Adenosine and GABA Neurotransmission in Rat Basal Forebrain" is currently in revision for publication in *Brain Structure and Function* where it has received a very favorable review. We anticipate submission of the revised version of this manuscript by 31 July 2014.

Progress - Task 4c: For Task 4c, we proposed to evaluate the effects of ALM and ZOL microinjections into the basal forebrain (BF) on sleep and wakefulness and on neurotransmitter release in the cerebral cortex (Cx). To date, no studies have reported the effect of central microinjections of hypnotics into the BF and their effects on sleep-wake behavior or neurotransmitters in brain. We proposed to use in vivo microdialysis and HPLC analyses to examine cortical adenosine (ADO), GABA, and glutamate (GLU) levels following BF microinjections of ALM, ZOL, or vehicle (VEH) combined with behavioral analyses. Task 4c was initiated mid-year in 2013 and, based on a power analysis, would require 70 rats to complete all of the n's per concentration (N=10 per group) to reach statistical significance ($p<0.05$) for this subtask. To date, a total of 23 rats contributed to the analyses for Task 4c. Several animals (N=6) had to be euthanized prior to entry into the protocol due surgical complications and thus did not contribute any data to the study. Each rat randomly received one of seven drug treatments (minimum 1 week apart with no more than two different drugs or dialysis attempts per animal) with parallel dialysis sampling in the cortex. Drug conditions (in μM) and experimental groups with the number of animals used in this study were as follows: VEH (N=8), ALM (1; N=7), ALM (0.3; N=7), ALM (0.1; N=3), ZOL (1; N=7), ZOL (0.3; N=5), and ZOL (0.1; N=4). One of seven drugs was microinjected into the animal 6 h into the dark period (ZT18), and 30 min dialysis samples (1 μL/min; CMA 12 probes) were collected to assess the effects of the drug on sleep-wake behavior and neurotransmitter levels in Cx.

Baseline EEG and EMG recordings were collected for 48 h via implanted telemetry devices concurrent with video recordings and microdialysis sampling. For all experiments, a microdialysis probe (2 mm length, 0.5 mm diameter, 20 kDa cutoff; CMA 12, CMA Microdialysis) was inserted into the cannula ~18 h prior to sample collection to allow for

neurotransmitter stabilization and perfused with aCSF at a rate of 1 µl/min. At the start of the experiment (3.5 h into the dark period; ZT 15.5), five 30 min baseline samples (1 µL/min flow rate; 30 µL total) were collected from freely-moving animals to assess basal levels of ADO, GABA, and GLU in conjunction with baseline EEG and EMG data. Following baseline collection, one of seven drug conditions was microinjected into the BF 6 h into the dark period (ZT 18) and then 12 additional 30 min samples were collected to assess the effects of the drug on sleep-wake and Cx neurotransmitter release (**Figure 14**). All samples in were collected in refrigerated fraction collectors at 4ºC and stored at -80ºC at the end of the experiment until analysis by HPLC.

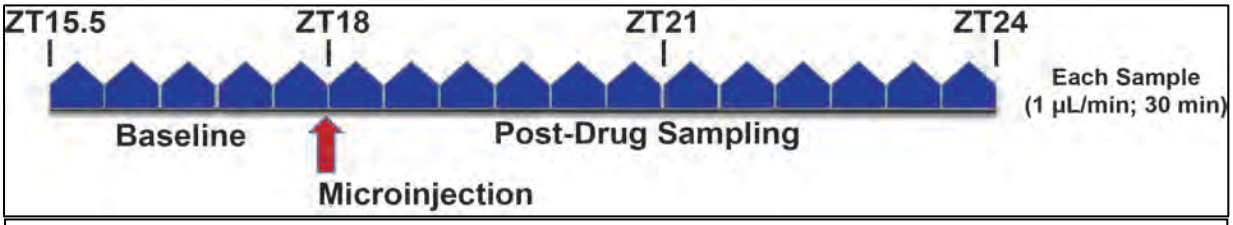

Figure 14. Schematic of the experimental design for Task 4c. Each blue pentagram box represents the collection of an individual microdialysis sample. Red arrow indicates time of microinjection at ZT18.

For HPLC quantification, the microdialysis samples were split into two vials for ADO (10 µL), GLU and GABA (20 µL) analysis. Quantification of ADO was measured by HPLC with UV detection. Samples (10 µL total volume) containing ADO were separated using mobile phase (10 mM monosodium phosphate, 7% acetonitrile, pH 4.50) pressurized through a U3000 isocratic pump with a flow of 0.8 mL/min. The dialysates flowed through a reversed-phase C18 column (150 mm ID x 4.6 mm, 2.6 µm, Phenomenex) and ADO was detected by UV at 254 nm. GLU and GABA dialysis content (20 µL total volume) were separated by HPLC with electrochemical detection (EC) using mobile phase (100 mM Na_2HPO_4, 22% MeOH, and 3.5% acetonitrile, pH 6.75) at a flow rate of 0.7 mL/min on a U3000 biocompatible isocratic pump. GLU and GABA were detected by precolumn derivatization using 2.2 mM O-phthalaldehyde and 0.8 mM 2-mercaptoethanol (β-ME) mixed by automation with the sample at 10°C for 2 min prior to injection into the HPLC. Separation was achieved through a reversed-phase C18 column (3.0 mm ID x 75 mm, 3 µm, Shiseido Capcell Pak) and electrically detected on a CouloChem III at the following potentials; E1; +250 mV, E2; +550 mV, Guard +650 mV at 45°C. Calibration curves for ADO and GLU/GABA were constructed using Chromeleon 6.8.0 software (Dionex, Corp., Sunnyvale, CA).

EEG and EMG were recorded via telemetry on a PC running Dataquest ART 3.1 (Data Sciences). All recordings were first screened for artifact and then manually scored offline in 10 s epochs as Wake, NREM, or REM sleep using NeuroScore 2.1 (DataSci™, St. Paul, MN). Any epochs that contained recording artifacts were tagged and excluded from subsequent analyses. Individual state data were quantified as time spent in each state per 30 min, 1 h, or 6 h. Latency to NR and REM onset for each animal was calculated from the time of drug injection. Bouts were defined as a minimum of 3 consecutive epochs of wake or NREM, and 2 consecutive epochs of REM sleep. NREM delta power was normalized to the average total spectral power for the 24 h baseline. Two way ANOVA and the Fisher's LSD multiple comparison test were used to determine any significant effects of drugs on neurotransmitter levels and sleep-wake behavior.

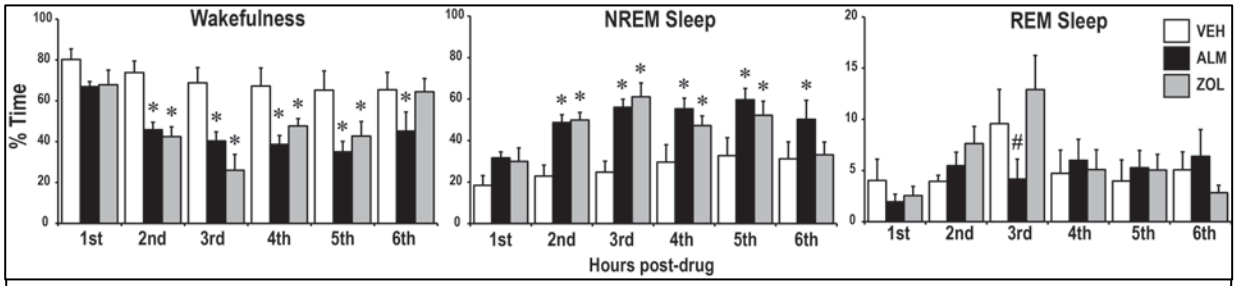

Figure 15. Effects of BF drug microinjections on percent time spent in behavioral state. ALM and ZOL caused a significant decrease ($p<0.05$) in % time spent in wake compared to VEH that persisted 6 h post-drug administration for ALM. An increase in NREM sleep with ALM and ZOL ($p<0.05$) was observed compared to VEH. * denotes significant difference from VEH. # denotes significant difference from ZOL.

Behavioral State Results: The results presented below are based on the data collected to date. There is wide variability within certain drug concentrations due to the low N for the ALM and ZOL groups, where VEH (N=8), ALM (1; N=7), ALM (0.3; N=7), ALM (0.1; N=3), ZOL (1; N=7), ZOL (0.3; N=5), and ZOL (0.1; N=4). As a result, there were insufficient N per group to perform the appropriate statistical comparisons both within and across the three drug conditions (VEH, ALM, and ZOL).

The data presented below show 3 conditions (VEH, ALM, and ZOL) for the highest dose of each drug concentration (ALM (10 ng/200 nl), ZOL (60 ng/200 nl)) and the effects on sleep-wakefulness and neurotransmitter release. BF microinjections of ALM and ZOL (**Figure 15**) significantly decreased the amount of time the animals spent in wakefulness compared to VEH controls (*$p<0.05$). This effect lasted for the 6 h post-drug administration.

Similarly, a significant increase in NREM sleep was observed in both ALM and ZOL conditions

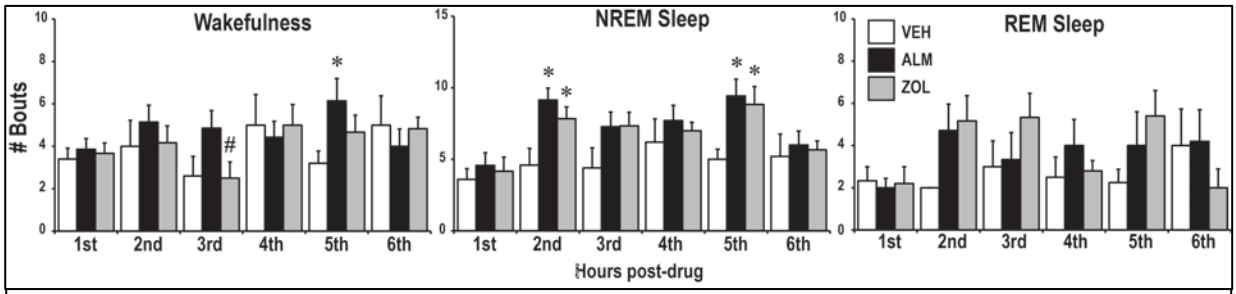

Figure 16. Effects of BF drug microinjections on bout number by behavioral state. # of Wake bouts was affected by ALM compared to VEH 5 h post drug-administration (*$p<0.05$). #$p<0.05$ denotes significant difference from ALM. # of NREM bouts also increased following ALM or ZOL at 2 and 5 h post-drug delivery (*$p<0.05$).

relative to VEH.

Wake bout frequency (**Figure 16**) was affected by ALM compared to VEH 5 h post drug-administration (*$p<0.05$). #$p<0.05$ denotes significant difference from ALM. An increase in NREM bout number was also observed with ALM and ZOL compared to VEH at 2 and 5 hrs post-drug administration (*$p<0.05$).

Although the mean duration of Wakefulness and REM sleep (**Figure 17)** was affected by ALM and ZOL compared to VEH during the 2nd, the 3rd and the 5th hours post drug (*$p<0.05$),

NREM sleep was unaffected. There was no effect on the latencies to either NREM or REM sleep.

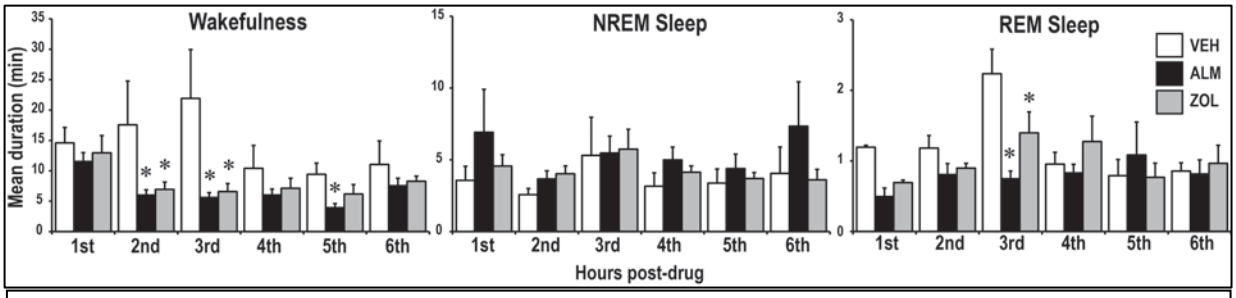

Figure 17. Effects of BF drug microinjections on mean duration for each behavioral state. Duration of Wake bouts were affected by ALM and ZOL at 2, 3 h and 5 h compared to VEH post drug-administration (*p<0.05).

Neurotransmitter Analyses and Results: Microdialysis samples were split into 2 vials and processed for both ADO, and GLU/GABA content. Two-way ANOVA revealed a significant drug x time interaction on cortical ADO release. *Post hoc* comparisons showed that ALM caused a significant increase ($p<0.05$) in cortical ADO (**Figure 18**) that persisted for hours throughout the experimental session. Microdialysis collection ended 6 h post-drug administration, at which time ADO appeared to be returning to baseline levels. On the other hand, cortical GABA levels were not significantly altered by BF microinjections of either ALM or ZOL compared to VEH. GABA levels remained stable throughout the 6 h experimental session post drug-administration. Interestingly, there appeared to be a general suppression of GLU release for several hours that began to return to basal levels by the 6th hour. *Post hoc* tests showed that ZOL caused a significant increase (#$p<0.05$) in cortical GLU relative to ALM at the end of the 6th hour post-drug. ADO is known to be inhibitory on excitatory glutamatergic neurons in vitro. These data suggest that cortical ADO may have a similar effect in vivo as our data show that ADO is significantly elevated for several hours following BF microinjections of ALM and the resulting

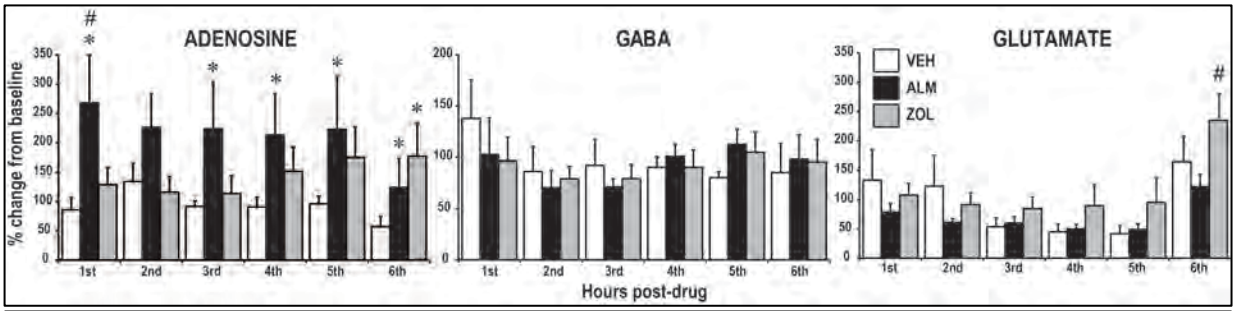

Figure 18. Effects of BF drug microinjections on neurotransmitter release in the cortex. Microinjections of ALM in the BF affected ADO release in the cortex (*p<0.05). ALM or ZOL had no effect on cortical GABA, and GLU appeared to be suppressed for several hours followed by a return to basal levels by the end of the hour 6 (#p<0.05 relative to ALM).

elevation of ADO in cortex may be providing that inhibitory influence on the surrounding glutamatergic neurons.

This study demonstrates that microinjection of ALM into the BF significantly increases the amount of time spent in NREM sleep at the expense of wakefulness (as seen by its effects on wake duration) similar to oral administration, as previously described (Dugovic et al., 2009;

Morairty et al., 2012). Microinjection of ZOL at the same dose also increased the percent of time spent in NREM sleep comparable to ALM. Microinjection of ALM increased the frequency of NREM bouts compared to VEH. Microinjection of ALM and ZOL decreased Wake bout duration compared to VEH. Microinjection of ALM promoted a sustained increase in ADO in the cerebral cortex over the 6 h recording during the dark period, whereas ZOL had no effect on ADO levels. Other cortical neurotransmitters such as GABA and GLU remained unaffected at the drug doses delivered to the BF. Consistent with these observations, the hypocretins (orexins) are known to modulate diverse physiological processes such as cognitive function and alertness.

The results of this study indicate that microinjection of ALM can induce sleep similar to oral delivery and facilitates the mechanisms that underlie the transition to normal sleep. ALM acts through blockade of post-synaptic Hcrt receptors, thereby disfacilitating excitation in the BF whereas ZOL, a benzodiazepine receptor agonist, affects a Cl^- channel on the $GABA_A$ receptor, resulting in hyperpolarization and general somnolence. In addition, systemic and local delivery of ALM (unlike ZOL) enhances ADO in both Cx and BF (manuscript under review) suggesting that the sleep-promoting effect of ADO may be via inhibition of Hcrt regulation of sleep-wakefulness.

Task 6: *Utilize optogenetics and in vivo physiology to compare the neural circuitry underlying ALM-induced vs. ZOL-induced sleep.*

6a. Determine whether activation of the Hcrt system is sufficient to induce arousal in the presence of ALM vs. ZOL.

6b. Determine whether ALM affects the activity of subcortical sites downstream from the Hcrt neurons.

6c. Determine how ALM and ZOL affect the activity of cortical neurons.

Technology Development: As described in last year's Progress Report, it was necessary to update the *In Vivo* Cellular Neurophysiology Laboratory before undertaking the experiments for Task 6 so that we could reliably perform optogenetic stimulation in freely-behaving mice across different recording sessions. This year, we made further technical refinements to our setup by utilizing commercially-produced bilateral fiber optic implants and a rotary joint that enabled light to be delivered to a mouse without constraining its movements (**Figure 19**). The bilateral implants depicted in Figure 19B are stereotactically implanted 1 mm above the hypocretin (Hcrt) field at 1mm lateral, 1.5 mm A-P and 3.5 mm D-V. To facilitate Aim 6b, we also acquired a microdrive that will allow us to position a Neuronexus array of 4 tetrodes that connects to the head stage of a Tucker Davis amplifier, thereby enabling us to record single neurons in deep brain areas while animals are freely moving (**Figure 20A**). To complement the electrophysiological measurements to be conducted in Aims 6b and 6c, we are also developing the capability to conduct calcium imaging in deep brain areas using the technology created by Inscopix, Inc. This technology consists of a miniature (~2 g) fluorescence microscope that can be mounted on the skull of a mouse and a microendoscope lens that can be implanted in the brain parenchyma. When coupled with genetically-encoded calcium indicators such as GCaMP6 expressed in local brain areas using a viral vector, the activity of hundreds of neurons can be visualized simultaneously (**Figure 20B and C**).

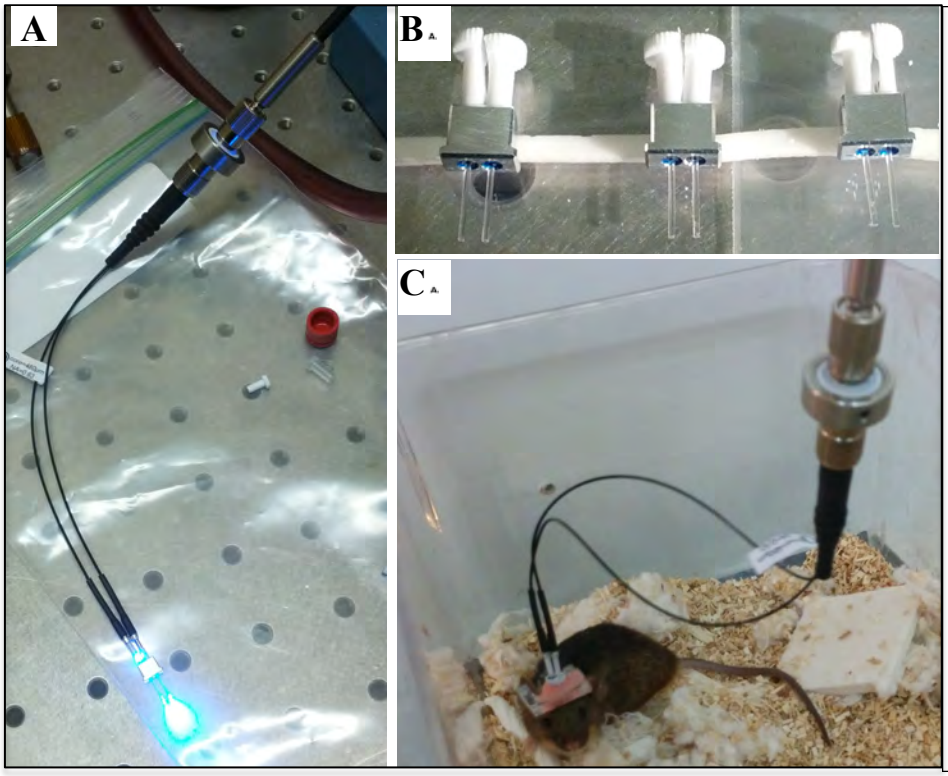

Figure 19. **A**) A LED based light engine (Lumencor) connects to a rotary joint which in turn connects to a patch cord that divides the light into two 0.5 mm fibers that can be attached to a bilateral fiber optic implant delivering 10 mW of light intensity at the output of each implanted fiber. **B**) Bilateral optical fiber implants (0.5 mm diameter). **C**) Implanted mouse in its home cage connected to the fiber optics for *in vivo* stimulation. The cage is placed over a DSI receiver that register EEG & EMG.

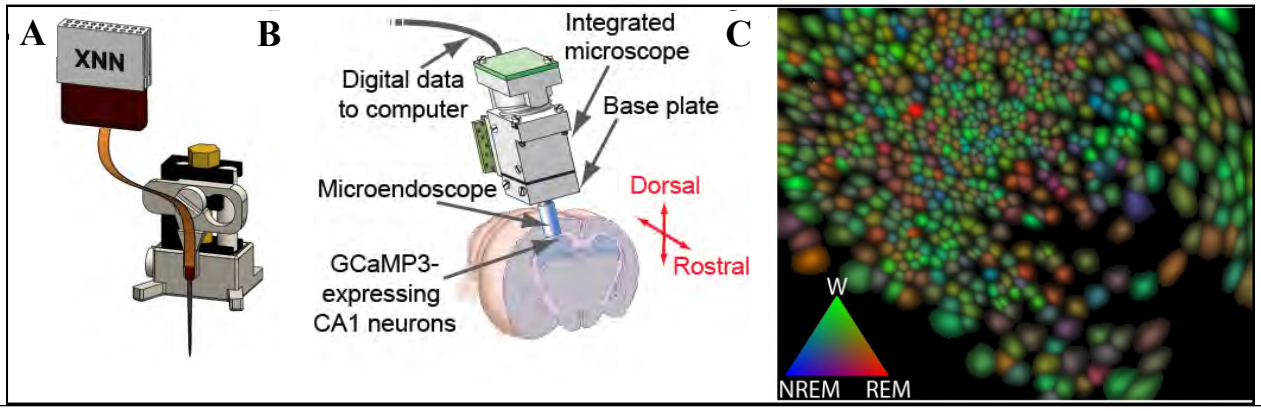

Figure 20. **A**) Schematic of the Neuronexus microdrive. **B**) Schematic of the Inscopix miniature fluorescence microscope. **C**) Example of hippocampal neurons identified with the Inscopix microscope and color-coded according to their activity rate during NREM sleep, REM sleep and Wake.

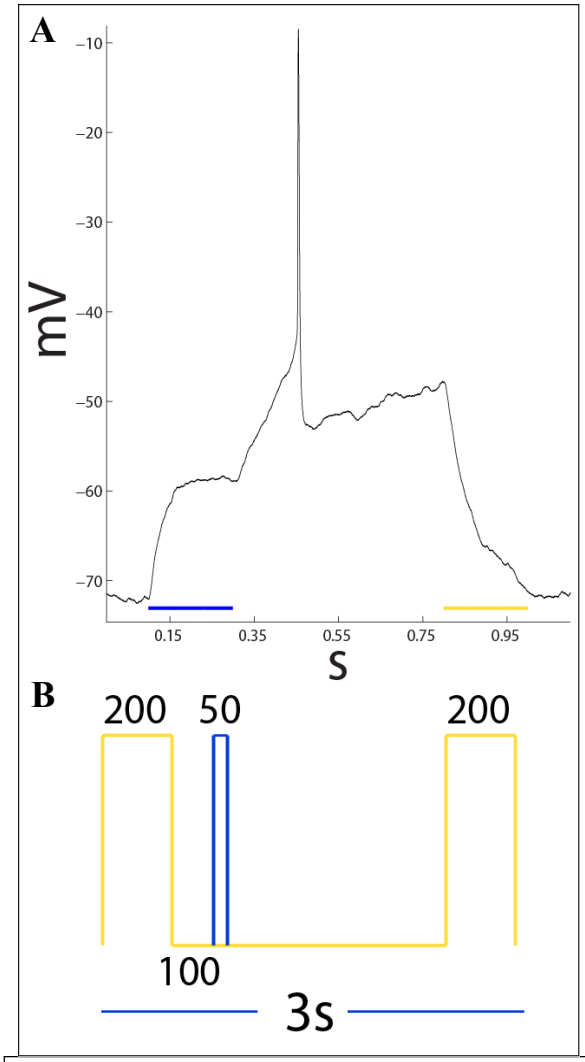

Figure 21. **A**. Depolarization of an Hcrt neuron from an *orexin-tTA; Tet-O ChR2(C128S)* mouse when illuminated by 100 ms pulse of blue and yellow light. **B**. For *in vivo* experiments, a 200 ms yellow pulse was followed by a 50 ms blue pulse and, 2650 ms later, by another 200 ms yellow pulse.

Progress - Task 6a: *In vitro* recordings of Hcrt neurons of *orexin-tTA; Tet-O ChR2(C128S)* mice show that a pulse of blue light depolarizes these neurons (**Figure 21**). *ChR2(C128S)* encodes a "step function opsin" (SFO) in which sodium channels remain open after blue light stimulation until closed by yellow light stimulation. Thus, in **Figure 21A**, V_m remains depolarized after the blue pulse until a yellow pulse closes the channels. To prevent desensitization of the SFO and to ensure that all channels were closed before delivering the pulse of blue light, we stimulated mice with a 50 ms pulse of blue light flanked by 200 ms yellow pulses (see **Figure 21B**) once every 4 min. To control for a possible effect of light illumination, we interleaved this stimulus pattern with pure yellow stimulation with the same pattern, i.e., the yellow 50 ms pulse was repeated once every 4 min. Thus, mice received either blue light flanked by yellow stimulation or a pure yellow stimulus every 2 min.

Mice (N=4) were implanted with the fiber optic implants described above and with a DSI telemetry transmitter to enable EEG and EMG recording. Recordings started at ZT4 and animals were dosed with either vehicle (Veh) or 300 mg/Kg almorexant (ALM) at ZT5. The volume injected was 0.15 ml IP. One hour later, optogenetic stimulation commenced at ZT6 for 1 h and an additional hour of EEG/EMG was recorded.

Figure 22 shows the raw EEG, EMG and light stimulus signal when mice were dosed either

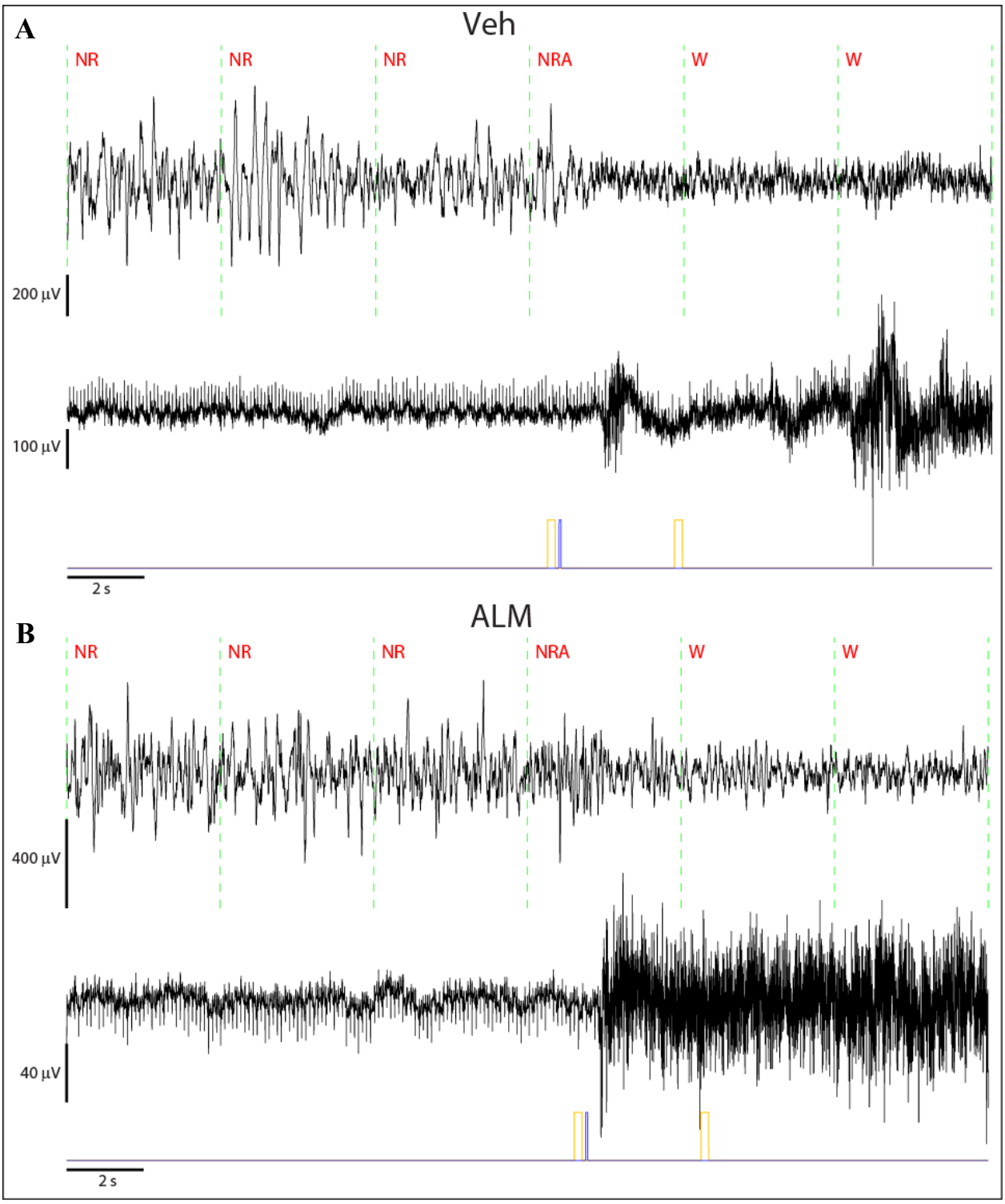

Figure 22. EEG/EMG recording of a mouse dosed with either Veh (**A**) or ALM (**B**) during optogenetic stimulation of Hcrt neurons. In each panel, the upper trace shows the EEG, middle trace is the EMG, and lower trace shows the light stimulus. 4 sec epochs were scored as either Wake (W), NREM sleep (NR) or NREM with artifact (NRA).

with Veh or ALM. In both treatment conditions, mice tended to awaken after the blue pulse

either briefly or for prolonged periods, as depicted in **Figure 22**. Note that, in both cases, the stimulation and awakening occurred within the same 4s epoch. When the stimulus was delivered during NREM sleep, animals woke up within 4 s after the blue pulse in 79% and 76% of the cases for Veh and ALM, respectively.

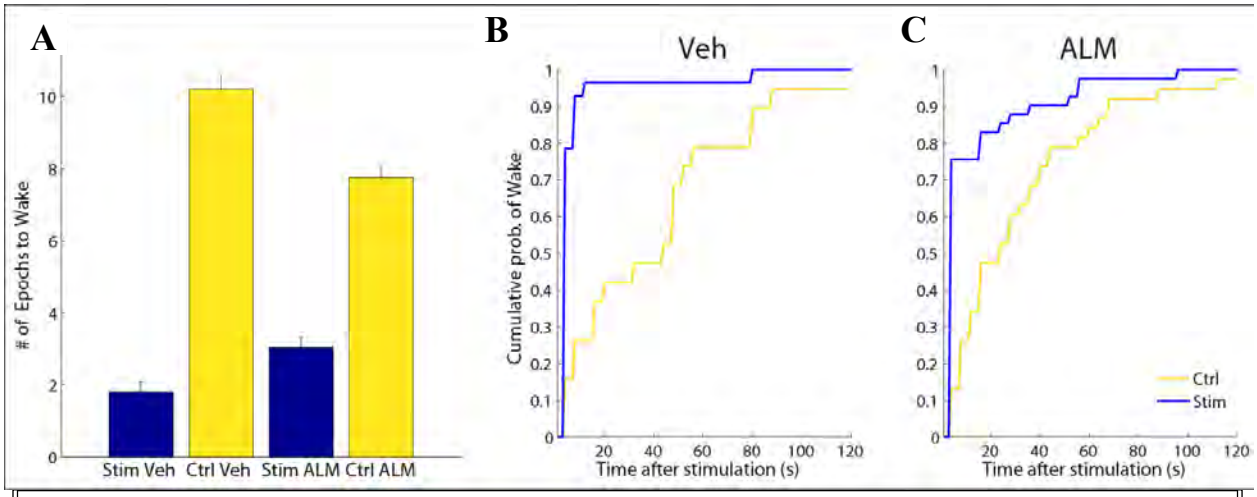

Figure 23. **A.** Number of 4s epochs elapsed after the light pulse until awakening occurred. Pulses were delivered during NREM sleep. **B, C.** Cumulative probability of wake after illumination when stimulated with blue light (stim) or with control (Ctrl) yellow light after Veh (**B**) or ALM (**C**) treatment.

Figure 23A shows the average number of epochs that elapsed from stimulation during NREM sleep until mice (N=4) awoke. In Veh-injected mice, the latencies to awakening were 1.8±0.3 and 10.2±0.4 epochs for 50 ms blue and yellow light pulses, respectively. For ALM-treated mice, the latencies to awakening were 3±0.3 and 7.8±0.4 epochs for 50 ms blue and yellow light pulses, respectively. Blue and yellow stimulation caused a significant difference (U test, $P<0.01$) but, within the same stimulus type, there was no difference between treatments in the 4 animals tested to date. **Figure 23B and C** show the cumulative probability of wake after stimulation with either blue (Stim) or yellow (Ctrl) light after Veh or ALM treatment. The fast rise of the Stim curve indicates that, in the large majority of cases, wake occurred in the next epoch after blue stimulation irrespective whether the mice had been treated with either Veh or ALM. After the Ctrl stimulus, the latencies to awakening are much longer, suggesting that illumination *per se* does not induce awakening. The fast time course of the awakening and the absence of a clear difference in the presence of the Hcrt antagonist ALM suggests that the observed arousals are mediated by glutamate release from Hcrt neurons projecting to wake-promoting areas such as the locus coeruleus (LC).To further characterize the changes in EEG upon Hcrt activation, we performed time-frequency analysis using 2 s windows. To calculate the average power in the standard EEG frequency bands, the FFT was shifted in 100 ms steps around the time of stimulation during NREM sleep. **Figure 24** shows that, for all bands below 60 Hz, optogenetic stimulation produced the decrease in spectral power amplitude that is expected during a transition from sleep to wake. This type of plot allows us to visualize the effect of Hcrt activation with greater time resolution in contrast to the coarse-grained analysis afforded by the 4s epoch classification shown in **Figure 22**. For both ALM and Veh, the change in spectral power is almost instantaneous at the time of stimulation, as reported when LC neurons were activated by direct optogenetic excitation (Carter et al 2010). This result supports the hypothesis that Hcrt neuron-mediated glutamate release evoked firing of LC neurons that, in turn, evoked a

general arousal.

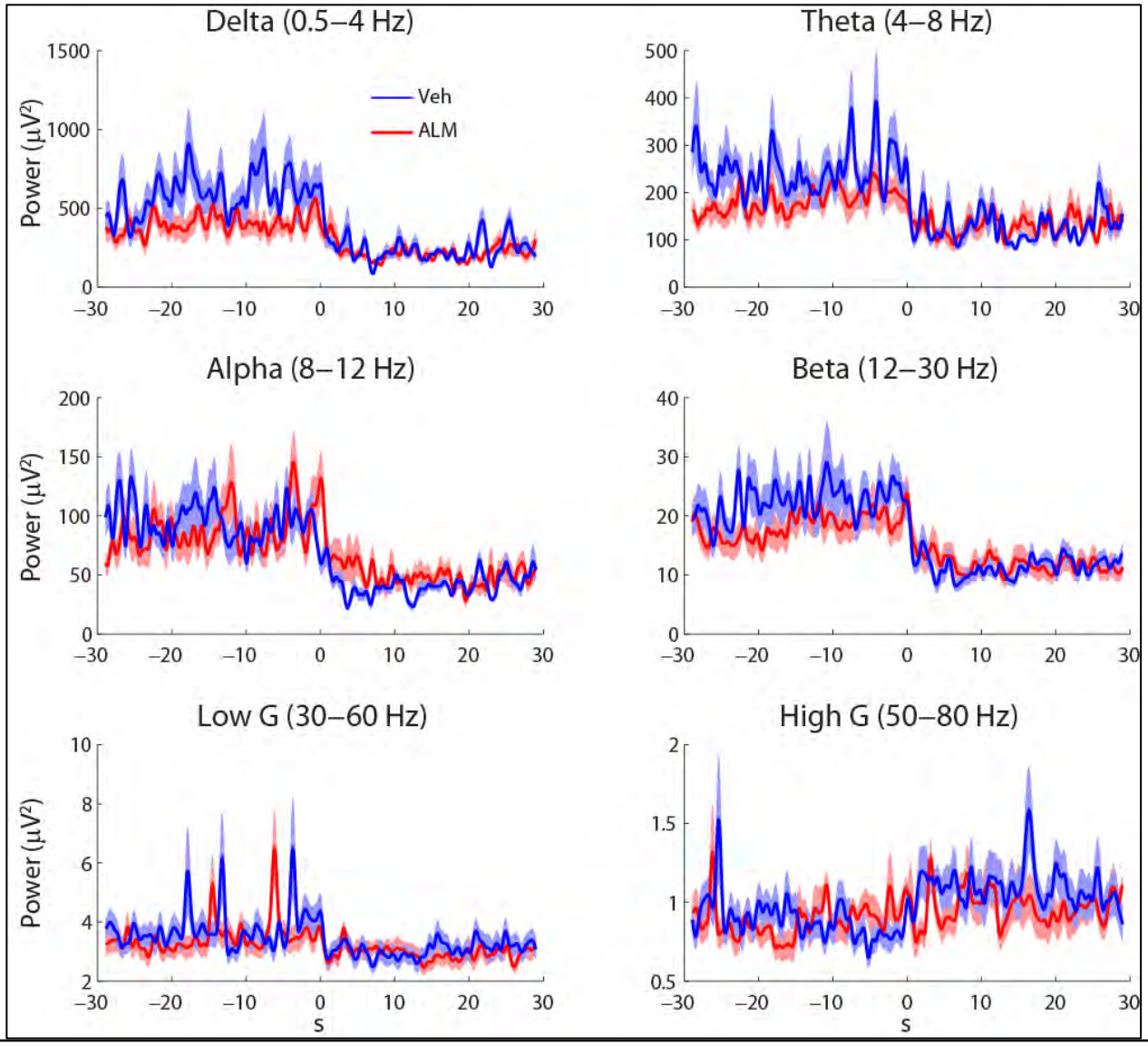

Figure 24. Time-frequency analysis for the average power in the standard EEG spectral bands. No significant difference was observed between ALM and Veh. Traces are centered on the time of blue light stimulation and the average power and SEM is depicted.

Plans for Year 6:

Task 2c: Data analysis for the rPVT study to be completed and manuscript to be submitted for publication.

Task 3a: The last few Fos double labeling experiments will be completed. Primary histology experiments have been completed for all markers, but staining needs to be repeated for a few animals for the ADA and Hcrt markers to ensure that all subregions have a sufficient number of cells scored to ensure an accurate analysis. Additionally, EEG/EMG analysis will be completed and the results correlated with Fos expression patterns. Once all experiments have been completed and analyzed, the results of this study will be prepared for publication.

Task 3b: Analysis for the second (locus coeruleus) lesion studies completed; a manuscript will be submitted for publication by 12/31/14. Data collection and analysis for the third (tuberomammillary nucleus) lesion study, which will require an additional 8 rats, will be completed.

Task 6a: We will complete Task 6a, optogenetic activation of Hcrt neurons in *orexin-tTA; Tet-O ChR2(C128S)* mice, and study the effects on sleep architecture at ZT 6 and 18 and after treatment with either Veh, ALM or Zol.

Tasks 6b and 6c: We will continue efforts to complete these tasks.

KEY RESEARCH ACCOMPLISHMENTS

- Aims 2a, 2b, and 4a completed and published.
- Manuscript describing results of Aims 3b.1 and 4b submitted and in revision.
- Data collection for Aims 2c and 3b.2 completed and data analysis ongoing.
- Data collection and analysis of Aim 3a continued.
- Data collection for Aims 3b.3, 4c and 6a initiated. In Task 6a, performed optogenetic excitation in freely behaving *orexin-tTA; Tet-O ChR2(C128S)* mice, which seem to tolerate the implant and the optogenetic stimulation without noticeable side effects.
- Initial results from Task 6a indicate that Hcrt neuron activation can cause a fast arousal that does not seem to be mediated by release of the Hcrt peptides as is not blocked in the presence of the Hcrt antagonist ALM.
- Implementation of a microdrive and a Neuronexus probe for recording multiunit activity in deep brain areas in Task 6b.
- Incorporated use of the Inscopix technology to record the activity of populations of neurons in specific brain areas.

REPORTABLE OUTCOMES

J. Vazquez-DeRose, A. Nguyen, S. Gulati, T. Mathew, and T. S. Kilduff (2013). Microinjections of the hypocretin antagonist almorexant vs. GABAergic agonist zolpidem in basal forebrain show differential effects on cortical adenosine levels in freely-moving rats. Program No. 478.11. *2013 Neuroscience Meeting Planner*. San Diego, CA: Society for Neuroscience, 2013. Online.

W. Lincoln, J Palmertson, T.C. Neylan, T.S. Kilduff, S.R. Morairty (2013). Zolpidem impairs attention/motivation in the rodent Psychomotor Vigilance Task more than almorexant. Program No. 658.24. *2013 Neuroscience Meeting Planner*. San Diego, CA: Society for Neuroscience, 2013. Online.

Morairty SR, Wilk A. Lincoln W, Neylan TC, and Kilduff TS. (2014). The hypocretin/orexin antagonist almorexant promotes sleep without impairment of performance in rats. *Front. Neurosci*. doi: 10.3389/fnins.2014.00003

Dittrich L, Morairty SR, Warrier D and Kilduff TS. Homeostatic sleep pressure is the primary factor for activation of cortical nNOS/NK1 neurons. *Neuropsychopharmalogy*, in press.

Vazquez-DeRose J, Schwartz MD, Nguyen AT, Warrier DR, Gulati S, Mathew TK, Neylan TC, and Kilduff TS. Hypocretin/orexin antagonism enhances sleep-related adenosine and GABA neurotransmission in rat basal forebrain. *Brain Structure and Function*, provisionally accepted.

CONCLUSION

During Year 5, one paper was published (Morairty et al. 2014), another has been accepted for publication (Dittrich et al., in press) and a third is in resubmission (Vazquez-DeRose et al., submitted). Data collection for Aims 2c and 3b.2 have been completed and data analysis ongoing; manuscripts will be written and submitted during Year 6. Data collection and analysis of Aim 3a in nearing completion; an abstract summarizing this work has been submitted for presentation at the 2014 Society for Neuroscience meeting. Data collection for Aims 3b.3, 4c and 6a have been initiated. The overall results obtained to date are consistent with the hypothesis that the hypocretin/orexin antagonist ALM produces less functional impairment than the benzodiazepine receptor agonist zolpidem (ZOL) because ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems.

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Morairty SR, Revel FG, Malherbe P, Moreau JL, Valladao D, Wettstein JG, Kilduff TS, Borroni E (2012). Dual hypocretin receptor antagonism is more effective for sleep promotion than antagonism of either receptor alone. *PLoS One* 7(7):e39131. doi: 10.1371/journal.pone.0039131.

APPENDICES

J. Vazquez-DeRose, A. Nguyen, S. Gulati, T. Mathew, and T. S. Kilduff (2013). Microinjections of the hypocretin antagonist almorexant vs. GABAergic agonist zolpidem in basal forebrain show differential effects on cortical adenosine levels in freely-moving rats. Program No. 478.11. *2013 Neuroscience Meeting Planner.* San Diego, CA: Society for Neuroscience, 2013. Online.

W. Lincoln, J Palmertson, T.C. Neylan, T.S. Kilduff, S.R. Morairty (2013). Zolpidem impairs attention/motivation in the rodent Psychomotor Vigilance Task more than almorexant. Program No. 658.24. *2013 Neuroscience Meeting Planner.* San Diego, CA: Society for Neuroscience, 2013. Online.

Morairty SR, Wilk A. Lincoln W, Neylan TC, and Kilduff TS. (2014). The hypocretin/orexin antagonist almorexant promotes sleep without impairment of performance in rats. *Front. Neurosci.* doi: 10.3389/fnins.2014.00003

Dittrich L, Morairty SR, Warrier D and Kilduff TS. Homeostatic sleep pressure is the primary factor for activation of cortical nNOS/NK1 neurons. *Neuropsychopharmacology*, in press.

Presentation Abstract

Program#/Poster#:	658.24/BBB16
Presentation Title:	Zolpidem impairs attention/motivation in the rodent psychomotor vigilance task more than almorexant
Location:	Halls B-H
Presentation time:	Tuesday, Nov 12, 2013, 4:00 PM - 5:00 PM
Topic:	++E.08.e Sleep: Systems and behavior
Authors:	**W. LINCOLN**[1], J. PALMERSTON[1], T. NEYLAN[2], T. KILDUFF[1], *S. R. MORAIRTY[1]; [1]Ctr. for Neurosci., SRI Int'l, MENLO PARK, CA; [2]UCSF/SFVAMC, San Francisco, CA
Abstract:	The dual hypocretin receptor (HcrtR) antagonist almorexant (ALM) has potent hypnotic actions but less is known about its effects on performance. Since Hcrt antagonists produce sleep by disfacilitation of wake-promoting systems whereas benzodiazepine receptor agonists (BzRAs) such as zolpidem (ZOL) induce sleep through a generalized inhibition of neural activity, we hypothesized that ALM would produce less functional impairment than ZOL. We have previously shown that rats tested in spatial reference memory or spatial working memory tasks in a water maze show no impairment following ALM whereas significant impairment was evident following ZOL. Here, we tested the effects of ALM and ZOL on the Rodent Psychomotor Vigilance Task (rPVT), a sensitive test of attention and motivation. 10 rats were implanted with telemetry devices for recording EEG and EMG. The effects of ALM and ZOL on attention/motivation administered in the middle of the active period were assessed at 2 sleep-promoting concentrations (30 & 100 mpk, po) following undisturbed and sleep deprived (SD, 6 h prior to dosing) conditions. 90 min following dosing, trained, water-restricted rats were placed in operant chambers with infrared detection beams in front of the water dispenser. rPVT testing consisted of a stimulus light (duration of 0.5 s) followed by a 3 s response period. The inter-trial interval varied between 3-7 s. Errors resulted in a cued 10 s “time out” period. Performance measures were 1) response latencies (RL), 2) correct responses (CR), 3) omissions (OM), and 4) premature errors (PE).

Impaired performance is indicated by increases in RL, OM and PE and decreases in CR.
SD had a relatively small but significant effect on performance following VEH: RL decreased (96.2%), CR decreased (95.5%) and OM increased (146.1%) while PE decreased (95.1%) following SD+90 min recovery compared to baseline. Following ALM at 30 mpk compared to VEH, OM and RL decreased (51.7 & 96.2%; indicative of increased performance) while CR decreased and PE increased (indicative of impaired performance). ZOL at 30 mpk decreased performance markedly: RL increased (131.3%), CR decreased (30.4%) and OM increased (724.1%) while PE decreased (36.9%) compared to VEH. However, performance decreased significantly following both drugs at 100 mpk, particularly with ZOL. Following ALM at 100 mpk, RL and OM increased (150.6 & 556.3%) and CR and PE decreased (42.8 & 58.0%). Following ZOL at 100 mpk, RL and OM increased (126.6 & 855.6%) and CR and PE decreased (9.2 & 26.0%).
These results are consistent with the hypothesis that less functional impairment results from HcrtR antagonism than from BzRA-induced inhibition.

Disclosures: **W. Lincoln:** None. **S.R. Morairty:** None. **J. Palmerston:** None. **T. Kilduff:** None. **T. Neylan:** None.

Keyword(s): ATTENTION

SLEEP DEPRIVATION

MOTIVATION

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Presentation Abstract

Program#/Poster#:	478.11/JJJ25
Presentation Title:	Microinjections of the hypocretin antagonist almorexant vs. GABAergic agonist zolpidem in basal forebrain show differential effects on cortical adenosine levels in freely-moving rats
Location:	Halls B-H
Presentation time:	Monday, Nov 11, 2013, 3:00 PM - 4:00 PM
Topic:	++E.08.c. Sleep: Molecular, cellular, and pharmacology
Authors:	***J. VAZQUEZ-DEROSE**[1], A. NGUYEN[1], S. GULATI[1], T. MATHEW[1], T. C. NEYLAN[2], T. S. KILDUFF[1]; [1]Ctr. for Neuroscience, Biosci. Div., SRI Intl., MENLO PARK, CA; [2]VA Med. Center/NCIRE, UCSF San Francisco, San Francisco, CA
Abstract:	Hypocretin (Hcrt-1 and Hcrt-2) peptides are well-known to regulate sleep and alertness and send projections to the basal forebrain (BF), an area critical for promoting wakefulness. The BF contains a heterogeneous mix of neurons that send diverse projections important for cortical arousal. Almorexant (ALM) is a dual Hcrt receptor antagonist that reversibly blocks signaling of both Hcrt receptors, whereas Zolpidem (ZOL) is a benzodiazepine receptor agonist affecting Cl- channels. Previous studies have shown that oral delivery of ALM elicits somnolence without cataplexy and, in rat, decreases active wake and increases the time spent in non-rapid eye movement (NREM) and (REM) sleep with differential effects on various neurotransmitter systems. To date, no studies have reported the effects of central microinjections of ALM or ZOL and its effect on behavior or transmitter levels in brain. We used in vivo microdialysis and HPLC analysis to examine cortical adenosine (ADO) levels following BF microinjections of ZOL (0.6 μg/0.2 μl), ALM (1.0 μg/0.2 μl), or VEH (aCSF) combined with behavioral analyses. Preliminary analyses revealed a significant main effect of drug on ADO levels. Post-hoc comparisons showed that ALM microinjected in to the BF (n=3 rats; * $p<0.05$) caused a significant increase in cortical ADO that lasted up to 6 h post microinjection compared to VEH control (n=3). Conversely, administration of ZOL (n=3) to the BF significantly decreased cortical ADO levels (# $p<0.05$) compared to VEH and ALM. These results provide novel evidence for dynamic

neurochemical changes underlying Hcrt modulation of sleep-wakefulness.

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The hypocretin/orexin antagonist almorexant promotes sleep without impairment of performance in rats

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The hypocretin receptor (HcrtR) antagonist almorexant (ALM) has potent hypnotic actions but little is known about neurocognitive performance in the presence of ALM. HcrtR antagonists are hypothesized to induce sleep by disfacilitation of wake-promoting systems whereas $GABA_A$ receptor modulators such as zolpidem (ZOL) induce sleep through general inhibition of neural activity. To test the hypothesis that less functional impairment results from HcrtR antagonist-induced sleep, we evaluated the performance of rats in the Morris Water Maze in the presence of ALM vs. ZOL. Performance in spatial reference memory (SRM) and spatial working memory (SWM) tasks were assessed during the dark period after equipotent sleep-promoting doses (100 mg/kg, po) following undisturbed and sleep deprivation (SD) conditions. ALM-treated rats were indistinguishable from vehicle (VEH)-treated rats for all SRM performance measures (distance traveled, latency to enter, time within, and number of entries into, the target quadrant) after both the undisturbed and 6 h SD conditions. In contrast, rats administered ZOL showed impairments in all parameters measured compared to VEH or ALM in the undisturbed conditions. Following SD, ZOL-treated rats also showed impairments in all measures. ALM-treated rats were similar to VEH-treated rats for all SWM measures (velocity, time to locate the platform and success rate at finding the platform within 60 s) after both the undisturbed and SD conditions. In contrast, ZOL-treated rats showed impairments in velocity and in the time to locate the platform. Importantly, ZOL rats only completed the task 23–50% of the time while ALM and VEH rats completed the task 79–100% of the time. Thus, following equipotent sleep-promoting doses, ZOL impaired rats in both memory tasks while ALM rats performed at levels comparable to VEH rats. These results are consistent with the hypothesis that less impairment results from HcrtR antagonism than from $GABA_A$-induced inhibition.

Keywords: hypocretins/orexins, cognitive impairment, memory impairment, hypnotics, water maze, spatial reference memory, spatial working memory, EEG

INTRODUCTION

Insomnia is a highly prevalent condition affecting 10–30% of the general population; (NIH, 2005; Roth, 2007; Mai and Buysse, 2008). Sleep loss and sleep disruption can lead to a degradation of neurocognitive performance as assessed by objective and subjective measures (Wesensten et al., 1999; Belenky et al., 2003; Lamond et al., 2007). Prescription sleep medications are often used to treat insomnia and obtain desired amounts of sleep. Presently, nonbenzodiazepine, positive allosteric modulators of the $GABA_A$ receptor such as zolpidem (ZOL) are the most widely prescribed hypnotic medications. Although known to induce sleep, these compounds have been shown to significantly impair psychomotor and memory functions in rodents (Huang et al., 2010; Uslaner et al., 2013; Zanin et al., 2013), non-human primates (Makaron et al., 2013; Soto et al., 2013; Uslaner et al., 2013) and humans (Balkin et al., 1992; Wesensten et al., 1996, 2005; Mattila et al., 1998; Mintzer and Griffiths, 1999; Verster et al., 2002; Storm et al., 2007; Otmani et al., 2008; Gunja, 2013). Such impairment can be particularly troubling when there is an urgent need for highly functional performance in the presence of drug such as with first responders, military personnel, and caregivers. Further, complex behaviors during the sleep period (e.g., eating, cooking, driving, conversations, sex) have been associated with these medications (Dolder and Nelson, 2008). Therefore, more effective hypnotics are needed that facilitate sleep that is easily reversible in the event of an unexpected awakening that demands unimpaired cognitive and psychomotor performance.

Recently, antagonism of the hypocretin (Hcrt; also called orexin) receptors has been identified as a target mechanism for the next generation of sleep medications (Brisbare-Roch et al., 2007; Dugovic et al., 2009; Whitman et al., 2009; Hoever et al., 2010, 2012a,b; Coleman et al., 2012; Herring et al., 2012; Winrow et al., 2012; Betschart et al., 2013). The Hcrt system is well known to play an important role in the maintenance of wakefulness (de Lecea, 2012; Inutsuka and Yamanaka, 2013; Mieda and Sakurai, 2013; Saper, 2013). Hcrt fibers project throughout the central nervous system (CNS), with particularly dense projections and receptor expression found in arousal centers including the locus

coeruleus, the tuberomammilary nucleus, dorsal raphe nuclei, laterodorsal tegmentum, pedunculopontine tegmentum, and the basal forebrain (Peyron et al., 1998; Marcus et al., 2001). The excitatory effects of the Hcrt peptides on these arousal centers is hypothesized to stabilize and maintain wakefulness. Therefore, blockade of the Hcrt system should disfacilitate these arousal centers, creating conditions that are permissive for sleep to occur.

The current study tests the hypothesis that the dual Hcrt receptor antagonist almorexant (ALM) produces less functional impairment than ZOL. The rationale that underlies this hypothesis is that ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems. We tested this hypothesis using tests of spatial reference memory (SRM) and spatial working memory (SWM) in the Morris Water Maze. Although the concentrations of ALM and ZOL administered prior to these tests were equipotent in hypnotic efficacy, the performance of rats treated with ALM were superior to that of rats treated with ZOL.

MATERIALS AND METHODS

ANIMALS

One hundred fifty three male Sprague Dawley rats (300 g at time of purchase; Charles River, Wilmington, MA) were distributed among the 12 groups as described in **Table 1**. All animals were individually housed in temperature-controlled recording chambers (22 ± 2°C, 50 ± 25% relative humidity) under a 12:12 light/dark cycle with food and water available *ad libitum*. All experimental procedures were approved by SRI International's Institutional Animal Care and Use Committee and were in accordance with National Institute of Health (NIH) guidelines.

SURGICAL PROCEDURES

Rats were instrumented with sterile telemetry transmitters (F40-EET, Data Sciences Inc., St Paul, MN) as previously described (Morairty et al., 2008, 2012; Revel et al., 2012, 2013). Briefly, under isoflurane anesthesia, transmitters were placed intraperitoneally and biopotential leads were routed subcutaneously to the head and neck. Holes were drilled into the skull at 1.5 mm anterior to bregma and 1.5 mm lateral to midline, and 6 mm posterior to bregma and 4 mm lateral to midline on the right hemisphere. Two biopotential leads used as EEG electrodes were inserted into the holes and affixed to the skull with dental acrylic. Two biopotential leads used as EMG electrodes were positioned bilaterally through the nuchal muscles.

IDENTIFICATION OF SLEEP/WAKE STATES

After at least 3 weeks post-surgical recovery, EEG, and EMG were recorded via telemetry using DQ ART 4.1 software (Data Sciences Inc., St Paul, MN). Following completion of data collection, the EEG, and EMG recordings were scored in 10 s epochs as waking (W), rapid eye movement sleep (REM), or non-rapid eye movement sleep (NREM) by expert scorers blinded to the treatments using NeuroScore software (Data Sciences Inc., St Paul, MN). Sleep latency was defined as the first 60 s of continuous sleep following drug administration. Recordings were started at Zeitgeber time (ZT) 12 (lights off) and continued until animals performed the water maze tests.

Table 1 | The number of rats tested for each of the 12 experimental groups.

Test	No SD			6 h SD		
	VEH	ALM	ZOL	VEH	ALM	ZOL
Reference memory	14	13	17	16	16	8
Working memory	11	12	12	12	11	11

SLEEP DEPRIVATION PROCEDURES

Animals were sleep deprived (SD) from ZT12-18 by progressive manual stimulation concurrent with EEG and EMG recording. The rats were continuously observed and, when they appeared to attempt to sleep, progressive interventions were employed to keep them awake: removal of cage tops, tapping on cages, placement of brushes inside the cage, or stroking of vibrissae or fur with an artist's brush.

DRUGS

Almorexant (ALM; ACT-078573), was synthesized at SRI International (Menlo Park, CA. USA) according to the patent literature. Zolpidem (ZOL) was a gift from Actelion Pharmaceuticals Ltd. For the SWM task, rats were dosed with ALM (100 mg/kg, p.o.), ZOL (100 mg/kg, p.o.) or vehicle (VEH; 1.25% hydroxypropyl methyl cellulose, 0.1% dioctyl sodium sulfosuccinate, and 0.25% methyl cellulose in water) at ZT18 and left undisturbed until time to perform memory tasks (see below). For the SRM task, most rats were also administered ALM, ZOL, and VEH p.o. at the concentrations above. However, one cohort of rats was administered drugs i.p. For these rats, ALM was administered at 100 mg/kg ($N = 6$), ZOL at 30 mg/kg ($N = 8$) and VEH ($N = 7$). ZOL is approximately 3X more potent i.p. than p.o.(Vanover et al., 1999) while ALM is equipotent through both routes of administration. Analysis of the sleep/wake data confirmed the equipotent effects of both drugs through both routes of administration at the concentrations tested.

WATER MAZE

All water maze (WM) tasks occurred in a pool 68″ in diameter and 25″ in depth, containing water at 24 ± 2°C made opaque by the addition of non-toxic, water soluble black paint and milk powder. Since all tests took place during the dark period, distinctive spatial cues were made of small "rice" lights colored blue, yellow, and green. Patterns of lights in distinct shapes (circle, square, diamond, "T" shape) were clearly visible from within the pool. Preliminary studies determined the minimum number of lights that were needed for learning to occur. A 10 cm diameter platform was submerged approximately 1 cm below the surface of the water in one of 6 locations (**Figure 1**). The platform location determined the orientation of the 4 quadrants used for analysis. Both WM tasks were similar to previous reports (Wenk, 2004; Ward et al., 2009).

TEST OF SPATIAL REFERENCE MEMORY

The acquisition phase occurred in one session consisting of 12–15 consecutive trials with a 60 s inter-trial interval. For each trial,

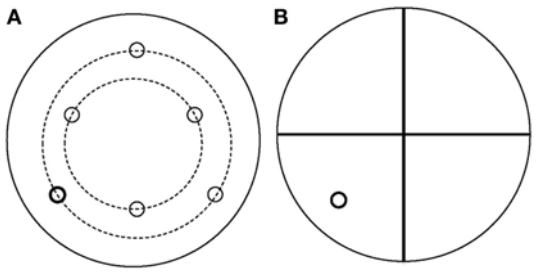

FIGURE 1 | Schematic of the water maze apparatus used for both spatial reference and spatial working memory tasks. (A) Schematic of the platform locations. **(B)** Example of quadrant orientations used for analysis used for the platform indicated in bold. Quadrant locations were always oriented so that the platform was central within a quadrant.

rats were placed in the WM facing the wall in one of three quadrants that did not contain the hidden platform. The location of the hidden platform remained constant across all trials. Rats were given 60 s to locate the platform. If the rats did not locate the platform within this period, they were guided to the platform location. When the rats reached the platform, they were allowed to remain on the platform for approximately 15 s before being placed in a dry holding cage for the next 60 s. This training sequence continued until the rats learned the task, typically 12–15 trials.

On the following day, rats were dosed with ALM, ZOL or VEH at ZT18 and a retention probe trial was performed 90 min later in which the rats were returned to the WM but the platform had been removed. A total of 40 rats were subjected to SD for 6 h prior to drug administration, and 42 were left undisturbed during this period (**Table 1**). Rats were started in the quadrant opposite the target quadrant and allowed to swim for 30 s. All trials were recorded by video camera and analyzed with Ethovision XT software (Noldus, Leesburg, VA). Test measures for the retention probe were time spent in target quadrant, latency to target quadrant, frequency of entrance into target quadrant, and total distance traveled. Swim speed was calculated to control for nonspecific effects.

TEST OF SPATIAL WORKING MEMORY

The SWM task consisted of 6 pairs of trials, one for each platform location (**Figure 1A**). In the first trial, a cued platform marked with a flag was placed in one of 6 positions in the WM. Rats were released facing the wall from one of the 3 quadrants not containing the platform and were allowed 120 s to locate the cued platform before the researcher guided the rats to the platform. This procedure provided all rats the opportunity to learn the platform location even if they did not find it on their own. After 15 s on the platform, the rats were removed from the WM and placed in a holding cage. The flag was then removed but the platform remained in the same location as in the first trial. Following a delay of 1, 5, or 10 min in the holding cage, the rats were placed back in the WM into one of the 2 quadrants that did not contain the platform and was not the starting quadrant during the first trial. Once the rats found the platform, they were removed after approximately 5 s and placed back in a holding cage for 10 min before a new pair of trials with a novel platform location was given. The order of delays was counterbalanced so that each rat was tested twice at 1, 5, or 10 min delays between the cued and hidden platforms. All trials were recorded by video camera and analyzed with Ethovision XT software (Noldus, Leesburg, VA). Test measures were time to locate the platform and the swim velocity during all tests.

STATISTICAL ANALYSIS

Statistical analyses were performed using SigmaPlot 12.3 (Systat Software Inc., San Jose, CA). Sleep/wake data (W, NREM, and REM time) were analyzed in 30 min bins and compared between drug groups using Two-Way mixed-model ANOVA on factors "drug group" (between subjects) and "time" (within subjects). SRM performance parameters (latency, duration and frequency in target quadrant, total distance traveled) were analyzed using a One-Way ANOVA. SWM performance measures (velocity, time to platform, percent found) by delay time were analyzed using Two-Way mixed-model ANOVA on factors "drug group" (between subjects) and "time" (within subjects). Significance levels were set at $\alpha = 0.05$. When ANOVA indicated significance, Bonferroni *t*-tests were used for *post hoc* analyses.

RESULTS

Drug concentrations were chosen to be equipotent at sleep promotion based on our previous experience (Morairty et al., 2012). Although ZOL produced a more rapid onset to sleep under both SD and undisturbed conditions (No SD: ZOL = 6.6 min, VEH = 32.2 min, ALM = 25.4 min; SD: ZOL = 5.9 min, VEH = 20.0 min, ALM = 15.5 min), ALM- and ZOL-treated rats slept equivalent amounts during the last hour before the WM test (**Figure 2**; No SD: ZOL = 69.4%, ALM = 62.3%, VEH = 37.6%; SD: ZOL = 69.6%, ALM = 71.5%, VEH = 52.0%).

TEST OF SPATIAL REFERENCE MEMORY

For all performance measures analyzed, rats treated with ZOL showed significant impairments while ALM- and VEH-treated rats were indistinguishable (**Figure 3**). Following ZOL, the latency to the target zone increased (No SD: ZOL = 14.1 s, VEH = 5.7 s, ALM = 5.8 s; SD: ZOL = 18.4 s, VEH = 4.2 s, ALM = 3.6 s) and the duration in the target zone (No SD: ZOL = 5.5 s, VEH = 8.4 s, ALM = 7.9 s; SD: ZOL = 4.8 s, VEH = 7.7 s, ALM = 7.8 s), frequency entering the target zone (No SD: ZOL = 1.2, VEH = 2.7, ALM = 2.5; SD: ZOL = 0.9, VEH = 2.8, ALM = 2.9) and the distance traveled (No SD: ZOL = 472 cm, VEH = 666 cm, ALM = 725 cm; SD: ZOL = 343 cm, VEH = 709 cm, ALM = 775 cm) all decreased compared to VEH and ALM-treated rats. ALM-treated rats did not differ from VEH-treated rats on any of these four measures. Performance in the SRM task was not significantly affected by 6 h SD for any measure within any group.

Swim patterns in the WM were different for ZOL-treated rats compared to VEH- and ALM-treated rats (**Figure 4**). Both VEH and ALM rats repeatedly swam across the WM and typically swam through the area where the hidden platform was present on the previous day (**Figure 4A**). In contrast, ZOL-treated rats primarily swam around the perimeter of the WM, a pattern typical of a rat during its first exposure to the WM.

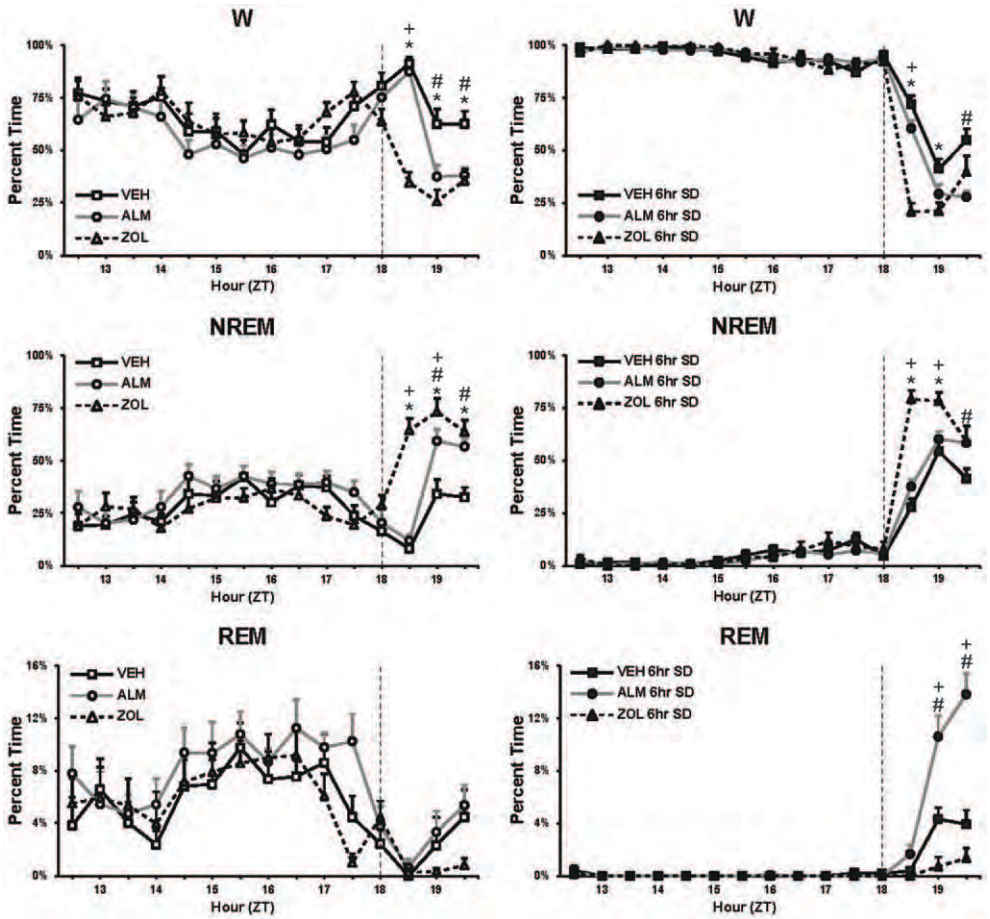

FIGURE 2 | Percent time spent in W, NREM, and REM during baseline (left panels) and during 6 h SD (right panels). The vertical line in each panel at ZT18 depicts the time of drug administration. At the end of the recording time displayed in these panels, rats were tested in the water maze. Note that, for the 60 min prior to testing (ZT19.5), the ALM and ZOL groups slept similar amounts. *, ZOL different from VEH; +, ZOL different ALM; #, ALM different from VEH; $p < 0.05$.

TEST OF SPATIAL WORKING MEMORY

ZOL-treated rats performed poorly in the SWM task compared to either VEH- or ALM-treated rats (**Figures 5**, **6**). ZOL-treated rats took longer to find the platform (No SD: ZOL = 43.4–47.3 s, VEH = 20.6–30.0 s, ALM = 22.5–30.7 s; SD: ZOL = 48.0–55.5 s, VEH = 26.9–31.0 s, ALM = 25.6–28.2 s) and swam more slowly (No SD: ZOL = 14.0–14.2 cm/s, VEH = 18.0–19.6 cm/s, ALM = 18.9–20.4 cm/s; SD: ZOL = 9.9–10.9 cm/s, VEH = 15.7–16.8 cm/s, ALM = 17.5–18.1 cm/s) than the VEH or ALM rats (**Figure 5**). These measures were not affected by increasing the delay from 1 to 5 min or 10 min for any of the 6 groups of rats.

The goal for the SWM task was to locate the platform. VEH- and ALM-treated rats found the platform the majority of the time in both SD and undisturbed conditions (83.3–100% for VEH and 79.2–87.5% for ALM; **Figure 6**). Conversely, ZOL-treated rats failed to find the platform most of the time (22.7–50.0% success rate). Interestingly, ZOL-treated rats also often failed to find the cued platform during the training phase of each pair of trials (**Figure 7**). The ZOL-treated rats in the baseline group found the cued platform 54.4% of the time while the SD ZOL-treated group were successful 53.8% of the time as compared to 98.6% for ALM-treated rats in the baseline group and 100% following SD and 100% of the time for all VEH-treated rats. A trend toward improved performance was observed with progressive trials in the ZOL-treated rats.

DISCUSSION

Though differing in the latency to induce sleep at the doses tested, ALM, and ZOL were equally effective at promoting sleep during the 90 min period prior to performance testing and both compounds significantly increased sleep compared to VEH. ALM-treated rats were indistinguishable from VEH-treated rats in their performance of both the SRM and SWM tasks. In contrast, ZOL caused significant impairments in both tasks. Specifically, in the SRM task, ZOL increased the latency to, the duration in, and the frequency of entering the target zone. In the SWM task, ZOL increased the time to find the platform, decreased the swim velocity and decreased the success rate in finding the platform. These results support the hypothesis that dual Hcrt receptor antagonism

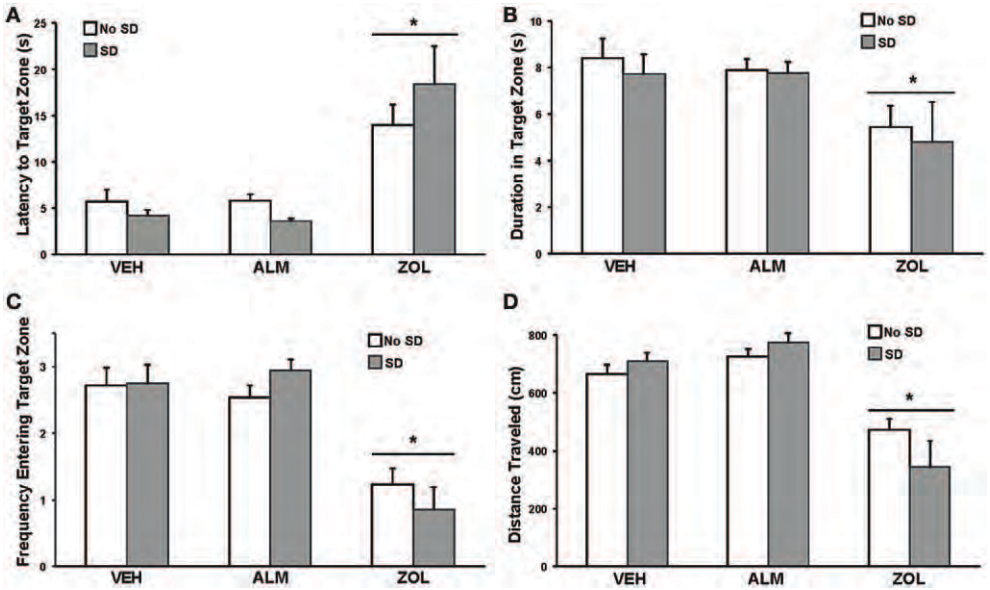

FIGURE 3 | Measures of performance in the spatial reference memory task. For all measures, ZOL-treated rats performed poorly compared to VEH- and ALM-treated rats. For all measures, the ALM-treated rats were indistinguishable from the VEH-treated rats. **(A)** Latency to the target zone. **(B)** Duration in the target zone. **(C)** Frequency entering the target zone. **(D)** Total distance traveled. For all measures, ANOVA revealed an effect of drug condition without an effect of SD. *, $p < 0.05$.

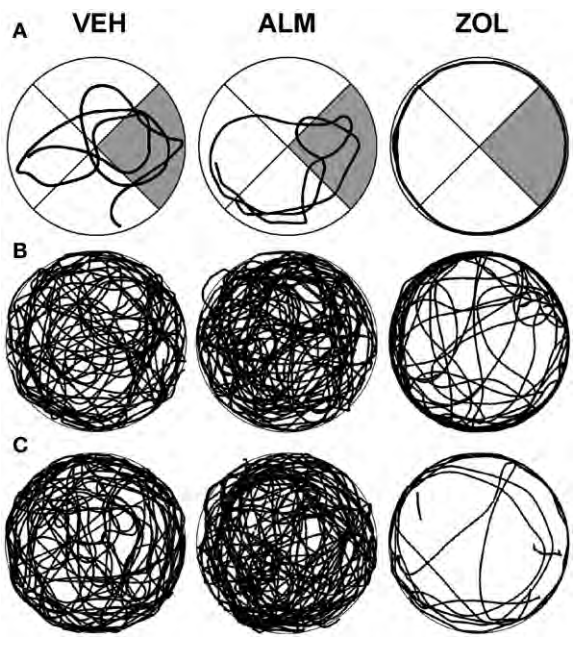

FIGURE 4 | Swim patterns during the spatial reference memory probe trials following VEH (left columns), ALM (center columns) and ZOL (right columns). (A) Examples of individual rats. The target quadrant is highlighted in gray. **(B)** Traces for all rats in the undisturbed condition. **(C)** Traces for all rats in the 6 h SD condition. Note that the searching pattern for VEH and ALM are similar while the pattern following ZOL remains primarily around the perimeter of the maze.

effectively promotes sleep without the functional impairments observed following $GABA_A$ receptor modulation.

An alternative explanation of the results obtained is that ZOL-treated rats were not motivated to perform the tasks rather than having memory/cognitive deficits. ZOL-treated rats had decreased distance traveled during the SRM task and decreased velocity during the SWM task, which could indicate a lack of motivation to escape the WM. Further, the lower success rate in finding the cued platform during the training trials for the SWM task could be interpreted as an absence of motivation to escape. However, ZOL rats did not simply float in the WM; they swam continuously, primarily circling the perimeter of the WM. As mentioned above, this swim pattern is typical of an untrained rat during its first exposure to the WM. Although not measured in this study, it is possible that the decreased distance traveled during the SRM task and decreased velocity during the SWM task are due to motor deficits produced by ZOL. This hypothesis is supported by previous studies that found prominent motor effects following ZOL administration (Depoortere et al., 1986; Steiner et al., 2011; Milic et al., 2012).

The SD protocol in these studies was included to assess whether moderate increases in sleep drive would exacerbate any cognitive deficits found following ALM or ZOL administration and also produce deficits in VEH-treated rats. While the primary active period of nocturnal rodents such as the rat is during the dark phase, rats still sleep approximately 30% of the time during this period and increasing wake duration during the dark period should create a mild sleep deficit (see **Figure 2**). Therefore, a portion of our experimental protocol involved SD during the 6 h of the dark period just prior to drug administration at ZT18.

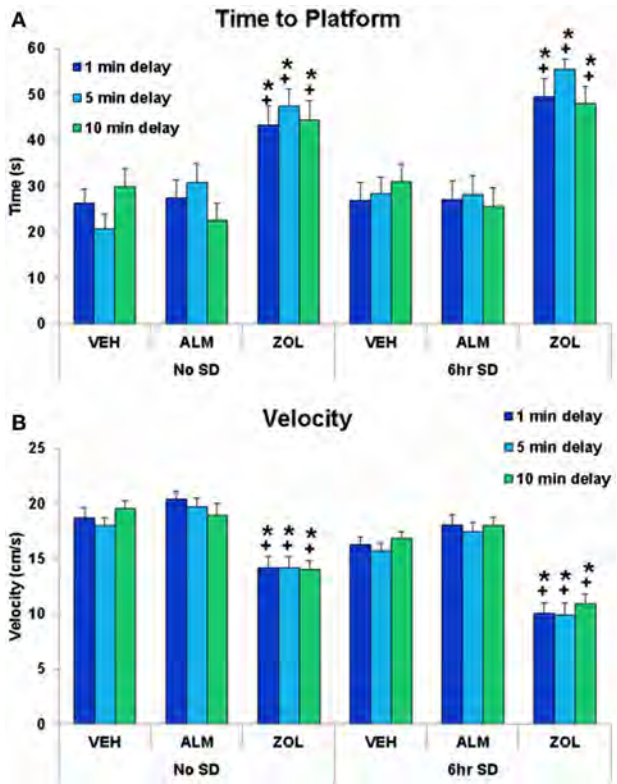

FIGURE 5 | The time to platform and the velocity swam during the spatial working memory task. (A) ZOL-treated rats found the platform significantly slower than VEH- or ALM-treated rats for all three delays following either undisturbed or SD conditions. The ALM-treated rats were not significantly different from VEH-treated rats for any condition. **(B)** ZOL-treated rats swam more slowly than either VEH- or ALM-treated rats. *, different from VEH; +, different from ALM; $p < 0.05$.

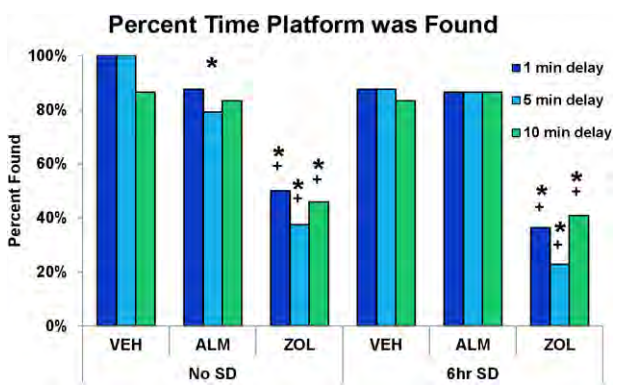

FIGURE 6 | Success rate in locating the platform during the test trials in the spatial working memory task. ZOL-treated rats found the platform significantly fewer times compared to VEH- or ALM-treated rats for all three delays and following both the undisturbed and SD conditions. In each trial, an individual rat either found or didn't find the platform; thus, there is no variation to represent as error bars in the graphs. *, different from VEH; +, different from ALM; $p < 0.05$.

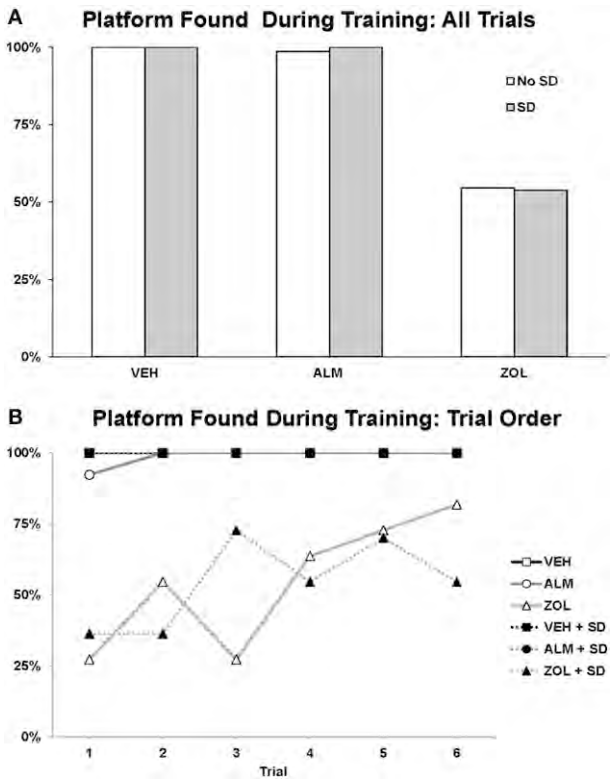

FIGURE 7 | Success rate in locating the platform during the training trials in the spatial working memory task. The platform was cued during these training trials by a flag. **(A)** The percentage of times the platform was found across all 6 training trials. **(B)** The percentage of times the platform was found trial by trial. Note that the ZOL rats tended to progressively improve across trials. In each trial, an individual rat either found or didn't find the platform; thus, there is no variation to represent as error bars in the graphs.

Although we did not find significant effects of SD vs. non-SD within any of the 3 dosing conditions, these results are likely due to the fact that we allowed the rats to sleep after drug administration until water maze testing began. This undisturbed period lasted only 60–90 min but provided an opportunity for the experimental subjects to recover from this mild sleep deprivation. If the SD were continued until testing, increased memory deficits might have been observed. Further studies are needed to determine whether this is indeed to case.

ZOL is a widely prescribed hypnotic medication that can be well-tolerated when taken as directed (Greenblatt and Roth, 2012). However, numerous adverse effects associated with ZOL usage have been reported including driving impairment (Verster et al., 2006; Gunja, 2013), effects on balance and postural tone (Zammit et al., 2008), interference with memory consolidation (Balkin et al., 1992; Wesensten et al., 1996, 2005; Mintzer and Griffiths, 1999; Morgan et al., 2010) and increased incidence of complex behaviors during sleep (Hoever et al., 2010). Some studies investigated the effects of daytime administration of ZOL and tested psychomotor function upon arousal from naps (Wesensten et al., 2005; Storm et al., 2007), a protocol which our experiments closely mimic. In these studies, ZOL or melatonin was

administered at either 10:00 or 13:00. Following a 1.5–2 h nap opportunity, subjects were awakened and required to perform a series of psychomotor and cognitive tests. Significant performance decrements were observed following ZOL in cognitive, vigilance and memory tasks while little to no decrements were found following melatonin. The results of ZOL administration on rat cognitive performance in the current study correlate well with these deficits found in humans.

In contrast, the high level of performance following ALM in both of our memory tasks suggests a high degree of safety at concentrations with hypnotic efficacy. Indeed, a recent study found no performance decrements in a variant of the WM SRM task at three-fold the concentration of ALM that we used (Dietrich and Jenck, 2010). Furthermore, another recent study found no effect of ALM at 300 mg/kg on motor function (Steiner et al., 2011). In humans, however, psychometric test battery assessment of the effect of ALM administered in the daytime found reductions in vigilance, alertness, and visuomotor and motor coordination at dose concentrations of 400–1000 mg (Hoever et al., 2010, 2012a). Notably, 400 mg ALM is within the therapeutic dose range required to improve sleep in patients with primary insomnia (Hoever et al., 2012b). Therefore, performance deficits following ALM occur within the range of hypnotic efficacy in humans. In one report, pharmacokinetic/pharmacodynamic modeling suggests that doses of 500 mg ALM and 10 mg ZOL are equivalent with respect to subjectively assessed alertness (Hoever et al., 2010). Since we find hypnotic efficacy to be achieved at roughly similar dose concentrations, there may be species differences in pharmacokinetic/pharmacodynamics of ALM and/or ZOL. While not uncommon, this makes direct translational interpretations of the present data more difficult. Regardless, in both rodents and humans, ALM appears to have a significantly better safety profile than ZOL with regards to cognitive/memory domains.

CONCLUSION

ALM and ZOL are effective hypnotics in multiple mammalian species (Brisbare-Roch et al., 2007; Hoever et al., 2010, 2012a,b; Morairty et al., 2012). They act through entirely different mechanisms of action, and their effects on cognition, psychomotor vigilance and memory are in stark contrast to one another. We found that at equipotent hypnotic concentrations, ZOL impaired SRM and SWM but ALM did not. These results support the hypothesis that antagonism of the Hcrt system can provide hypnotic efficacy without the impairments found by inducing sleep through $GABA_A$ modulation.

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Homeostatic Sleep Pressure is the Primary Factor for Activation of Cortical nNOS/NK1 Neurons

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Abbreviated title: Sleep pressure activates cortical nNOS neurons

ABSTRACT

Cortical interneurons, immunoreactive for neuronal nitric oxide synthase (nNOS) and the receptor NK1, express the functional activity marker Fos selectively during sleep. NREM sleep "pressure" is hypothesized to accumulate during waking and to dissipate during sleep. We reported previously that the proportion of Fos^+ cortical nNOS/NK1 neurons is correlated with established electrophysiological markers of sleep pressure. Since these markers covary with the amount of NREM sleep, it remained unclear whether cortical nNOS/NK1 neurons are activated to the same degree throughout NREM sleep or whether the extent of their activation is related to the sleep pressure that accrued during the prior waking period. To distinguish between these possibilities, we used hypnotic medications to control the amount of NREM sleep in rats while we varied prior wake duration and the resultant sleep pressure. Drug administration was preceded by 6 h of sleep deprivation ("high sleep pressure") or undisturbed conditions ("low sleep pressure"). We find that the proportion of Fos^+ cortical nNOS/NK1 neurons was minimal when sleep pressure was low, irrespective of the amount of time spent in NREM sleep. In contrast, a large proportion of cortical nNOS/NK1 neurons was Fos^+ when an equivalent amount of sleep was preceded by sleep deprivation. We conclude that, while sleep is necessary for cortical nNOS/NK1 neuron activation, the proportion of cells activated is dependent upon prior wake duration.

INTRODUCTION

Although the functions of sleep remain controversial, one of the strongest arguments for its fundamental importance is its homeostatic regulation. Sleep homeostasis refers to compensatory increases in sleep amount, sleep consolidation and/or sleep intensity that occur in response to a period of extended wakefulness (Borbely and Achermann, 2000). In mammals and birds, sleep intensity, usually assessed by measuring the spectral power of the EEG in the delta frequency range (0.5-4.5 Hz) during non-rapid eye movement sleep (NREM), is used as an index of the hypothetical sleep "pressure" that has accumulated during wakefulness (Borbely and Achermann, 2000; Rattenborg *et al*, 2009).

Despite the widespread occurrence of sleep homeostasis among animal species, our understanding of the underlying mechanisms is incomplete. Current hypotheses implicate sleep factors such as adenosine or cytokines that accumulate during waking and increase the propensity and depth of sleep (Coulon *et al*, 2012; Krueger *et al*, 2008; Landolt, 2008; Porkka-Heiskanen and Kalinchuk, 2011; Szymusiak and McGinty, 2008). Sleep factors are thought to act by inhibiting wake-promoting neurons (Porkka-Heiskanen and Kalinchuk, 2011; Rainnie *et al*, 1994), but may also act directly on the cerebral cortex (Clinton *et al*, 2011; Szymusiak, 2010). We have recently described a population of cortical GABAergic interneurons that is specifically activated during sleep (Gerashchenko *et al*, 2008; Pasumarthi *et al*, 2010). These neurons are identified by colocalized immunoreactivity for neuronal nitric oxide synthase (nNOS) and the substance P (SP) receptor NK1 (Dittrich *et al*, 2012). Activation of these neurons, assessed by immunoreactivity for the functional activity marker Fos, correlates with time spent in NREM sleep as well as with NREM delta power (Gerashchenko *et al*, 2008; Morairty *et al*, 2013). Prior wake duration "dose-dependently" increased the proportion of Fos-labeled nNOS neurons when rats were subjected to 2h, 4h, or 6h of sleep deprivation (SD) followed by a 2h recovery sleep (RS) opportunity (Morairty *et al*, 2013). Based on these observations, we have suggested that cortical nNOS/NK1 neurons are inhibited by wakefulness and activated by sleep pressure (Kilduff *et al*, 2011).

In our previous studies, we increased sleep pressure by increasing the amount of

prior wakefulness, which resulted in a compensatory increase in the amount of NREM sleep during RS. Therefore, we could not distinguish whether the time spent in NREM sleep or the magnitude of sleep pressure produced by prolonging wakefulness was the primary factor driving Fos expression in cortical nNOS/NK1 neurons. Fos expression typically reflects neuronal activity occurring during the 1-2 h prior to sacrifice (Hoffman and Lyo, 2002; Zangenehpour and Chaudhuri, 2002). If cortical nNOS/NK1 neurons are uniformly activated throughout NREM sleep and inactive during wakefulness, Fos expression in these neurons should depend on the time spent in NREM during the 1-2 h preceding sacrifice and thus would only indirectly correlate with measures of sleep pressure. Here, we sought to distinguish between these alternatives by dissociating the occurrence of NREM from the magnitude of sleep pressure using hypnotic medications to pharmacologically control NREM sleep duration under conditions in which the prior sleep/wake history was varied. To ensure that our results were not drug-specific, we utilized hypnotics with different mechanisms of action: the dual hypocretin/orexin receptor antagonist almorexant (ALM) and the $GABA_A$ receptor modulator zolpidem (ZOL). We find that, when time spent in NREM sleep is held constant, the proportion of cortical nNOS/NK1 cells activated is dependent upon prior sleep/wake history and that Fos expression in cortical nNOS/NK1 neurons reflects time kept awake (and, presumably, the accompanying sleep pressure) more robustly than any other parameter of NREM sleep.

MATERIALS AND METHODS

Animals

A total of 39 male Sprague-Dawley rats were studied. Animals were housed in separate cages in temperature-controlled recording chambers (20-24^{o}C, 30-70% relative humidity) under a 12/12 light/dark cycle with food and water available *ad libitum*. The weights at experiment were 587g ± 63 (mean ± SD). All experimental procedures involving animals were approved by SRI International's Institutional Animal Care and Use Committee and were in accordance with National Institute of Health (NIH) guidelines.

Surgical procedures

Surgical procedures involved implantation of sterile telemetry transmitters (F40-EET, Data Sciences Inc., St Paul, MN) as previously described (Morairty *et al*, 2013; Morairty *et al*, 2008; Morairty *et al*, 2012). Briefly, transmitters were placed intraperitoneally under isoflurane anesthesia. Biopotential leads were routed subcutaneously to the head and neck. EEG electrodes were placed epidurally 1.5 mm anterior to bregma and 1.5 mm lateral to midline, and 6 mm posterior to bregma and 4 mm lateral to midline on the right hemisphere. EMG leads were positioned bilaterally through the nuchal muscles.

Identification of Sleep/Wake States and Sleep/Wake Data Analyses

Behavioral state determinations and data analyses were conducted as previously described (Morairty *et al*, 2013; Morairty *et al*, 2012). After at least 3 weeks post-surgical recovery, EEG and EMG were recorded via telemetry using DQ ART 4.1 software (Data Sciences Inc., St Paul, MN). Following completion of data collection, the EEG and EMG recordings were scored in 10 s epochs as waking, rapid eye movement sleep (REM), or non-rapid eye movement sleep (NREM) by expert scorers who examined the recordings visually using NeuroScore software (Data Sciences Inc., St Paul, MN). For calculation of bout durations, a bout was defined as consisting of a minimum of two consecutive epochs of a given state and ended with any single state change epoch. EEG spectra were analyzed with a fast Fourier transform algorithm using a Hanning Window without overlap (NeuroScore software, Data Sciences Inc., St Paul, MN) on all epochs without artifact. For comparisons of EEG spectra, average spectra of a specific state were normalized to the average spectra of the respective state during a 6 h baseline recording (Zeitgeber time 0-6, or ZT0-ZT6). For calculation of NREM EEG delta power (NRD), the mean of the power between 0.5–4.5 Hz of the averaged NREM spectra was calculated and normalized to the respective value of the 6 h baseline recording. NRD energy (NRDE) was calculated by multiplying the time (h) spent in NREM sleep by the normalized NRD power.

Detection of individual slow waves was adapted from (Vyazovskiy *et al*, 2007). Raw EEG was bandpass filtered (0.5-4.5 Hz) using the bandpassfilter.m function from

the FieldTrip toolbox (http://www.ru.nl/neuroimaging/fieldtrip) in MATLAB (Mathworks, Natick, MA). The first positive peak after a zero crossing was identified as a single slow wave. The slope was approximated as a straight line between that peak and the last negative peak preceding the zero crossing. All slopes from artifact-free NREM epochs were averaged for each rat. Slopes were normalized to the average NREM slopes from the respective baseline recordings.

Sleep deprivation procedures

Animals were continuously observed while EEG and EMG were recorded and, when inactive and appeared to be entering sleep, cage tapping occurred. When necessary, an artist's brush was used to stroke the fur or vibrissae. After ZOL, it was sometimes necessary to touch rats to keep them awake.

Experimental Protocol

The rats were assigned to six groups: (1) VEH with low sleep pressure (*n*=6); (2) VEH with high sleep pressure (*n*=7); (3) ZOL with low sleep pressure (*n*=6); (4) ZOL with high sleep pressure (*n*=7); (5) ALM with low sleep pressure (*n*=6); and (6) ALM with high sleep pressure (*n*=7). Dosing occurred at ZT12, 100 mg/kg p.o. in 2 ml/kg for both drugs. Perfusion occurred at ZT14 for VEH and ZOL groups and at ZT14.5 for ALM groups due to its longer latency to sleep onset (Black *et al*, 2013; Morairty *et al*, 2012; Morairty *et al*, 2014). Rats in the high sleep pressure conditions were sleep deprived during the 6 h prior to dosing (Figure 1).

Immunohistochemical procedures

Rats were killed with an overdose of euthanasia solution i.p. (SomnaSol, Butler-Schein, Dublin, OH) and transcardially perfused with heparinized phosphate buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were postfixed overnight in the same fixative and then immersed in 30% sucrose in phosphate buffered saline until they sank. Coronal brain sections were cut at 40 µm thickness. Double immunohistochemistry for Fos and nNOS was performed on serial sections of rat brain as described previously (Gerashchenko *et al*, 2008; Pasumarthi *et al*, 2010). Sections

were first incubated overnight with rabbit anti-Fos antibody (1:4000-5000, sc-52, Santa Cruz Biotechnology, Dallas, TX), then with biotinylated donkey anti-rabbit antibody (1:500, Jackson ImmunoResearch, West Grove, PA), followed by avidin-biotinylated horseradish peroxidase complex (1:200, PK-6100, Vector Laboratories, Burlingame, CA), and nickel-enhanced 3,3' diaminobenzidine (SK4100, Vector Laboratories) for a black reaction product. nNOS neurons were stained in the same sections by overnight incubation in rabbit-nNOS antibody (1:2000, 61-7000, Invitrogen, Camarillo, CA), followed by biotinylated donkey anti-rabbit antibody and avidin-biotinylated horseradish peroxidase complex, and visualized with NovaRED (SK-4805, Vector Laboratories) for a red-brown reaction product.

Cell Counting

Single-labeled nNOS and double-labeled Fos^{+}/nNOS cells were counted in one hemisection each at 1.4 mm anterior, 0.5 mm posterior, and 3.0 mm posterior to bregma (Paxinos *et al*, 1999). The percentage of nNOS neurons expressing Fos was calculated as described previously (Gerashchenko *et al*, 2008; Pasumarthi *et al*, 2010). Micrographs for publication were taken at 200x magnification on a Leica DM 5000B microscope (Leica Microsystems, IL) with a Microfire S99808 camera (Optronics, CA) in Stereoinvestigator (MBF Bioscience, Williston, VT). Adjustments of brightness, color or contrast were applied to the whole image and performed in Photoshop (Adobe Systems, San Jose, CA).

Statistics

Statistical tests were performed using Excel (Microsoft, Redmond, WA), MATLAB and R (R Foundation for Statistical Computing). For each studied variable, we used Mann-Whitney U-tests to test whether it significantly distinguished between the high sleep pressure and low sleep pressure groups within the same drug treatment condition. The NREM bout duration histograms (Figure 2) were tested with 2-way permutation ANOVA (Manly, 2007) with 5000 iterations of the factors "bout duration" and "sleep pressure". If an interaction was found, the sleep pressure conditions were compared for each bout duration using Holm-Sidak corrected t-tests. For comparison of EEG power spectra, we

first performed 2-way permutation ANOVA with 5000 iterations with factors “frequency bin” and “sleep pressure”. If interactions were found, the source of the interaction was evaluated through bin-by-bin (0.122 Hz) uncorrected t-tests between the sleep pressure conditions. Only coherent changes that affected a range of frequencies were considered potentially meaningful, whereas isolated bins with significant changes were ignored. To determine how strongly different physiological parameters distinguished the high vs. low sleep pressure groups, we calculated Hedges’ g (difference of the means divided by pooled standard deviation) and the 95% confidence intervals as an effect size measure (Hedges and Olkin, 1985). Since Hedges’ g is a parametric measure, data were first transformed to achieve a normal distribution. The percent time in NREM and the %Fos^{+}/nNOS neurons were arcsine transformed. NREM bout durations, NRD, slow wave slopes (each normalized by respective baseline values), and NRDE were $\log_{10}$ transformed. Normal distributions after data transformation were verified visually using normal probability plots. To test whether Hedges’ g for %Fos/nNOS was significantly different from Hedges’ g for any other variable, g was first transferred to Fisher’s z (Borenstein *et al*, 2009). The z for %Fos/nNOS was then compared to z for every other variable (Meng *et al*, 1992) and the p-values were Holm-Sidak-corrected for multiple comparisons.

RESULTS

Sleep time can be dissociated from sleep/wake history using hypnotics

After experimental manipulation of sleep pressure as illustrated in Figure 1, the VEH-dosed rats showed the expected influence of waking history on sleep propensity: whereas undisturbed rats showed an increased time spent awake beginning at lights off (ZT12), rats that were sleep deprived during the preceding 6 h showed strongly reduced wakefulness at the same time of day (Figure 2A). ALM decreased the time spent awake in both groups but, at the dose used, the sleep deprived rats showed a stronger reduction of wakefulness than rats that were undisturbed for the 6 h preceding dosing, indicating an additive effect of ALM and sleep pressure (Figure 2B). In contrast, ZOL

caused a strong reduction of wakefulness irrespective of the preceding wake history (Figure 2C). Since we previously found that Fos expression in rat cortical nNOS neurons is dependent on NREM time during the 90 min preceding transcardial perfusion (Morairty *et al*, 2013), we focused on that time window for the following analyses. Figure 2D-F depicts the time each rat spent in wake, NREM, and REM during the 90 min immediately preceding sacrifice. Whereas the time spent in wake, NREM and REM differed between the low and high sleep pressure groups treated with either VEH or ALM, these physiological parameters did not differ between the groups treated with ZOL indicating a decoupling between prior sleep/wake history and vigilance states with ZOL treatment.

ZOL disrupts established measures of sleep pressure

Given the results in Figure 2D-F, we evaluated whether the high and low sleep pressure groups could be distinguished after ALM and ZOL treatment using four established measures of sleep pressure: NREM bout duration, NREM delta power (NRD), NREM delta energy (NRDE), and slow wave slopes. NREM bout duration frequency histograms were shifted towards longer bout durations in the sleep deprived groups (Figure 2G-I). Although there was a significant interaction between the factors "bout duration" and "sleep pressure" following VEH ($F_{5,55}$=6.74, p=0.007) and ALM ($F_{5,55}$=4.60, p=0.0008), this interaction did not reach statistical significance for ZOL ($F_{5,55}$=2.28, p=0.056). Accordingly, the average NREM bout durations were longer for sleep deprived than undisturbed rats following VEH (p=0.002) and ALM (p=0.008), but not for ZOL (p=0.073; Figure 2J).

As expected, the NREM EEG power spectra showed an elevated power in the delta range in the high sleep pressure group for VEH-dosed rats (Figure 3B). The wake and NREM spectra for ALM-dosed rats resembled those of VEH-dosed rats, including the increased NREM delta power in the high sleep pressure group (Figure 3C, D). Following ZOL, wake and NREM spectra were strongly altered compared to the corresponding baseline recordings, as indicated by the deviations from the basal value 1 in Figure 3E, F. In contrast to VEH and ALM, neither a main effect of "sleep pressure" nor an interaction of "frequency bin" and "sleep pressure" was found following ZOL for

either wake or NREM spectra, indicating that spectral power did not depend on prior sleep/wake history. (REM spectra are not shown because, in some groups, too little REM occurred to calculate representative spectra). Figure 3G, H depicts normalized NRD (0.5-4.5 Hz) and NRDE for each rat. Both measures significantly distinguished the high vs. low sleep pressure groups following VEH ($p=0.005$ for NRD, $p=0.005$ for NRDE) as well as ALM ($p=0.022$ for NRD, $p=0.001$ for NRDE) treatment, whereas no difference was found following ZOL ($p=1$ for NRD, $p=0.63$ for NRDE).

Lastly, we measured the average slopes of individual EEG slow waves during NREM. As expected, the slow wave slopes were steeper for sleep deprived rats than for undisturbed rats following VEH (Figure 4B). This difference was preserved after ALM (Figure 4C) but not after ZOL (Figure 4D). Consequently, the average slow wave slope was significantly greater in the high sleep pressure than in the low sleep pressure group following VEH ($p=0.008$) and ALM ($p=0.001$) but not following ZOL ($p=0.366$; Figure 4E).

Fos expression in nNOS neurons depends on prior sleep/wake history and resultant sleep pressure

To determine whether the percentage of Fos^+ cortical nNOS neurons depends on prior sleep/wake history or only on NREM time during the 90 min preceding sacrifice, we performed double-immunohistochemistry for Fos and nNOS. As depicted in Figure 5A-E, sleep deprived rats showed higher levels of %Fos/nNOS than undisturbed rats irrespective of drug treatment. Consequently, %Fos/nNOS significantly distinguished between the high vs. low sleep pressure conditions following VEH ($p=0.001$), ZOL ($p=0.001$) and ALM ($p=0.001$; Figure 5E). Notably, the separation between conditions was absolute after each drug treatment, i.e., there were no overlapping data points.

Fos/nNOS is the best indicator of sleep/wake history and resultant sleep pressure

To determine which physiological measure was most closely related to prior sleep/wake history, we quantified the effect sizes (Hedges' g) for the difference between the high vs. low sleep pressure groups for each of the parameters measured in the present study

(see Methods). For each drug treatment, Hedges' g was greatest for %Fos/nNOS (Figure 5F-H). Following VEH, %Fos/nNOS separated the sleep pressure groups significantly better than bout duration, NRD, or NRDE (Figure 5F) and better than all parameters following ALM (Figure 5G).

DISCUSSION

These results demonstrate that the extent of activation of cortical nNOS/NK1 neurons is determined by prior sleep history. When sleep pressure is assumed to be low, cortical nNOS neurons are largely inactive (Figure 5A-E) even in the presence of high amounts of NREM sleep as illustrated by the ZOL group in Figure 2E.

%Fos/nNOS depends on sleep/wake history

Using hypnotic treatment, we were able to dissociate time spent asleep during the 90 min before sacrifice from the prior sleep/wake history. Following ZOL, neither time spent in wake, NREM, nor REM differed between rats that were sleep deprived and rats that were left undisturbed for the preceding 6 h. Nonetheless, in ZOL-treated rats, the proportion of Fos^+ cortical nNOS neurons was significantly greater in the high sleep pressure than the low sleep pressure group. We conclude that cortical nNOS neurons are not activated simply by the occurrence of NREM sleep, rather, the %Fos/nNOS depends on the magnitude of sleep pressure that has accumulated during the time preceding sleep onset. Together with our previous studies (Gerashchenko *et al*, 2008; Morairty *et al*, 2013), these results demonstrate that cortical nNOS neurons are responsive to homeostatic sleep drive.

We found that the proportion of Fos^+ cortical nNOS neurons was a better indicator of prior sleep/wake history than total time spent in NREM, average NREM bout duration, NREM delta power, or the average slope of NREM slow waves (Figure 5F-H). Following ZOL, %Fos/nNOS was the only measure that significantly distinguished between the low and high sleep pressure groups. This observation makes it unlikely that Fos expression in cortical nNOS neurons is downstream of any of these variables

(e.g., driven by slow wave activity), although experimental confirmation will depend on the ability to selectively manipulate cortical nNOS/NK1 neurons.

In agreement with our previous findings (Morairty *et al*, 2014), the hypnotic efficacy of ALM and ZOL was comparable at the doses used in the present study. Nonetheless, the same dose of ALM produced different amounts of sleep in the high sleep pressure and low sleep pressure groups. This result is consistent with the view that ALM removes a wake-inducing input – Hcrt tone – whereas ZOL actively inhibits neuronal activation. Rather unspecific neuronal inhibition might bias the system towards sleep, whereas Hcrt antagonism might just impair the ability to stay awake in the presence of endogenous sleep pressure. A more detailed comparison will be needed to test if this is indeed a qualitative difference between the drugs or an effect of non-equivalent doses. Nonetheless, despite increased NREM sleep, ALM did not increase levels of Fos in cortical nNOS neurons in the low sleep pressure condition, which is consistent with the results obtained from the ZOL experiment.

nNOS/NK1 neurons and NREM delta power

The finding that, following ZOL, NRD did not differ between sleep deprived and undisturbed rats despite the pronounced difference in %Fos/nNOS between groups was surprising, since we have previously found that activation of these neurons may facilitate NRD (Morairty *et al*, 2013). Therefore, we had expected that increased Fos expression in cortical nNOS neurons would coincide with increased NRD in conditions when total NREM time did not differ. Given the pronounced effects of ZOL on the EEG, it is conceivable that ZOL masked the effects of cortical nNOS neuron activation on the NREM EEG. The low frequencies of the NREM power spectra were conspicuously increased following ZOL irrespective of sleep pressure (Figure 3D). Since cortical nNOS neurons are GABAergic (Kubota *et al*, 2011), the $GABA_A$ modulator ZOL might act directly on the downstream targets of these neurons. This interpretation is in agreement with the finding that the sleep pressure-dependent modulation of the NREM EEG was not impaired by the Hcrt antagonist ALM, since sleep deprived rats showed significantly elevated NRD. Based on Hedges' g, the difference in NRD between high

and low sleep pressure conditions was not smaller following ALM than VEH treatment (Figure 5).

Although the downstream targets of sleep-active cortical nNOS neurons have not yet been identified, these neurons are present in all cortical areas (Vincent and Kimura, 1992), form long-range cortico-cortical projections (Tomioka *et al*, 2005), and appear to be the origin of a dense nNOS-positive fiber network (Vincent and Kimura, 1992; Yousef *et al*, 2004) that is suited for producing a near simultaneous NO signal throughout a large cortical volume (Philippides *et al*, 2005). Thus, it seems likely that cortical nNOS neurons may exert a widespread effect on the cortex during sleep. This inference is supported by our recent finding that nNOS KO mice show deficits in regulation of delta power and consolidation of NREM sleep (Morairty *et al*, 2013). However, direct and specific experimental manipulation of these neurons will be necessary to determine the specific effects on cortical activity patterns.

Regulation of nNOS/NK1 neurons

While NREM sleep appears to be a permissive state for activation of cortical nNOS neurons, we demonstrated here that the magnitude of activation of these cells during NREM depends on prior sleep/wake history. The mechanism by which prior wake time is linked to activation of cortical nNOS neurons is of great interest, since it could provide insight into how the accumulation of sleep pressure is tracked by the brain. A better understanding of this mechanism could prove relevant for facilitating restorative sleep or combating pathological sleepiness. The integration of time spent awake might occur at the level of the nNOS/NK1 neurons themselves. Locally accumulating sleep factors, such as adenosine and cytokines, might activate these neurons (Kilduff *et al*, 2011). Another such factor could be Substance P (SP). mRNA levels of the gene coding for SP are increased in the cortex by sleep deprivation (Martinowich *et al*, 2011). Cortical nNOS neurons co-express the SP receptor NK1 and are strongly and directly activated *in vitro* by SP (Dittrich *et al*, 2012).

Fos expression in cortical nNOS/NK1 neurons remains minimal as long as rats are kept awake, irrespective of accrued sleep pressure (Morairty *et al*, 2013). Therefore, if

integration of sleep pressure indeed occurs at the level of cortical nNOS neurons, a wake-related inhibitory input must be assumed that prevents activation of these cells before sleep onset. This view is congruent with the model we have presented previously (Kilduff *et al*, 2011).

Alternatively, the integration of sleep pressure might occur upstream of cortical nNOS neurons. In this scenario, cortical nNOS neurons would receive activating input only during NREM, the magnitude of which depending on the sleep/wake history. In order to identify the mechanisms by which sleep pressure is linked to activation of cortical nNOS neurons, it will be critical to characterize the anatomical and neurochemical inputs to these cells.

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FIGURE LEGENDS

Figure 1. Experimental design. Sleep pressure increases during the active phase and decreases during the inactive phase. Rats were dosed at ZT12 (first vertical dashed line), when sleep pressure is lowest. We assume that sleep pressure increases slowly, if at all, during hypnotic-induced sleep; therefore, the corresponding curve remains low after dosing. To increase sleep pressure but keep the circadian conditions identical, rats in a second group were subjected to 6 h of sleep deprivation starting at ZT6 and dosed at ZT12. Rats in both conditions were perfused 2 or 2.5h after dosing (second vertical dashed line). Black and white bars indicate light conditions.

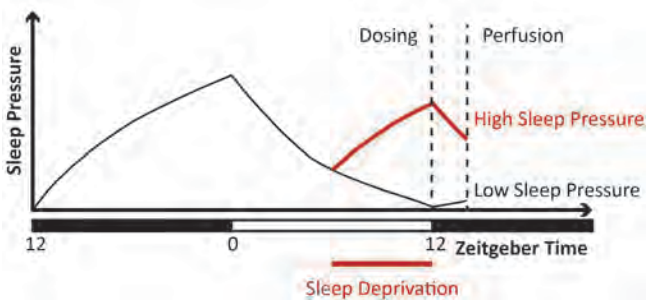
Figure 2. Time spent awake and asleep before sacrifice. **A-C**. Time spent awake between ZT6 and the time of transcardial perfusion. High sleep pressure groups were sleep deprived from ZT6-ZT12, low sleep pressure groups were left undisturbed so that the amount of sleep pressure differed. Lighting conditions are indicated below the panels. Dosing occurred at ZT12. **D-F**. Time spent in wake, NREM, and REM during the 90 min preceding sacrifice. After ZOL dosing, the time spent in any state did not differ between the sleep pressure conditions. Horizontal lines indicate group medians. *p<0.05, U-test. **G-I**. NREM bout duration frequency histograms during the 90 min preceding sacrifice following VEH (G), ALM (H) and ZOL (I) dosing. *p<0.05, Holm-Sidak test after significant interaction in permutation ANOVA. **J**. Average NREM bout durations. *p<0.05, U-test

Figure 3. Spectral analyses. Wake (**A, C, E**) and NREM (**B, D, F**) EEG power spectra for the 90 min preceding sacrifice were normalized by the respective baselines (ZT0-6). Interactions of factors "frequency" and "sleep pressure" (permutation ANOVA) are indicated for each panel. The degrees of freedom are 491 and 5401 for all interactions. The p-values for *post hoc* uncorrected bin-by-bin t-tests are indicated below the spectra. Following VEH and ALM dosing, increased sleep pressure coincided with increased NREM delta power (arrows) whereas, after ZOL dosing, NREM delta power was increased irrespective of sleep pressure. **G**. Average NREM delta power (NRD) during the 90 min preceding sacrifice. **H**. NREM delta energy (NRDE) during 90 min preceding sacrifice. Both NRD and NRDE distinguished between the high and low sleep pressure groups following VEH and ALM but not following ZOL dosing. Horizontal lines indicate group medians. *p<0.05, U-test

Figure 4. Slopes of NREM EEG slow waves during the 90 min preceding sacrifice. **A**. The raw EEG trace (top) was bandpass filtered in the slow wave range 0.5-4.5 Hz (bottom). Positive (green) and negative peaks (red) were identified. A straight line between a negative and a positive peak encompassing a zero crossing was defined as the slope of the respective slow wave. **B, C, D**. Average slow waves ± SEM for the experimental groups. **E**. Average NREM slow wave slopes. The slopes distinguished between the low and high sleep pressure groups following VEH and ALM but not following ZOL dosing. Horizontal lines indicate group medians. *p<0.05, U-test

Figure 5. Fos expression in cortical nNOS neurons depends on sleep pressure. **A-D**. Example micrographs of Fos/nNOS double immunohistochemistry. Following both hypnotics, nNOS neurons were single-labeled (arrows) in low sleep pressure conditions (A, C). In the high sleep pressure conditions (B, D), many nNOS neurons were double-labeled for Fos (black triangles) irrespective of the drug treatment. Scale bar indicates 50 µm. **E**. Proportion of Fos^+ cortical nNOS neurons. Note that %Fos/nNOS

completely separated all high sleep pressure groups from the respective low sleep pressure groups. Horizontal lines indicate group medians. *p<0.05, U-test. **F-H**. Effect sizes for the difference between low and high sleep pressure groups using six different measures. Data for each of the six parameters listed on the abscissa were transformed to obtain normal distributions. For each variable, Hedges' g ± 95% confidence interval was calculated as an effect size for the difference between the two groups following VEH (F), ALM (G), and ZOL (H) treatment. Following each drug treatment, the strongest effect was seen for %Fos/nNOS. ***** indicates significantly ($p<0.05$) smaller g than that for %Fos/nNOS after Holm-Sidak correction for multiple comparisons.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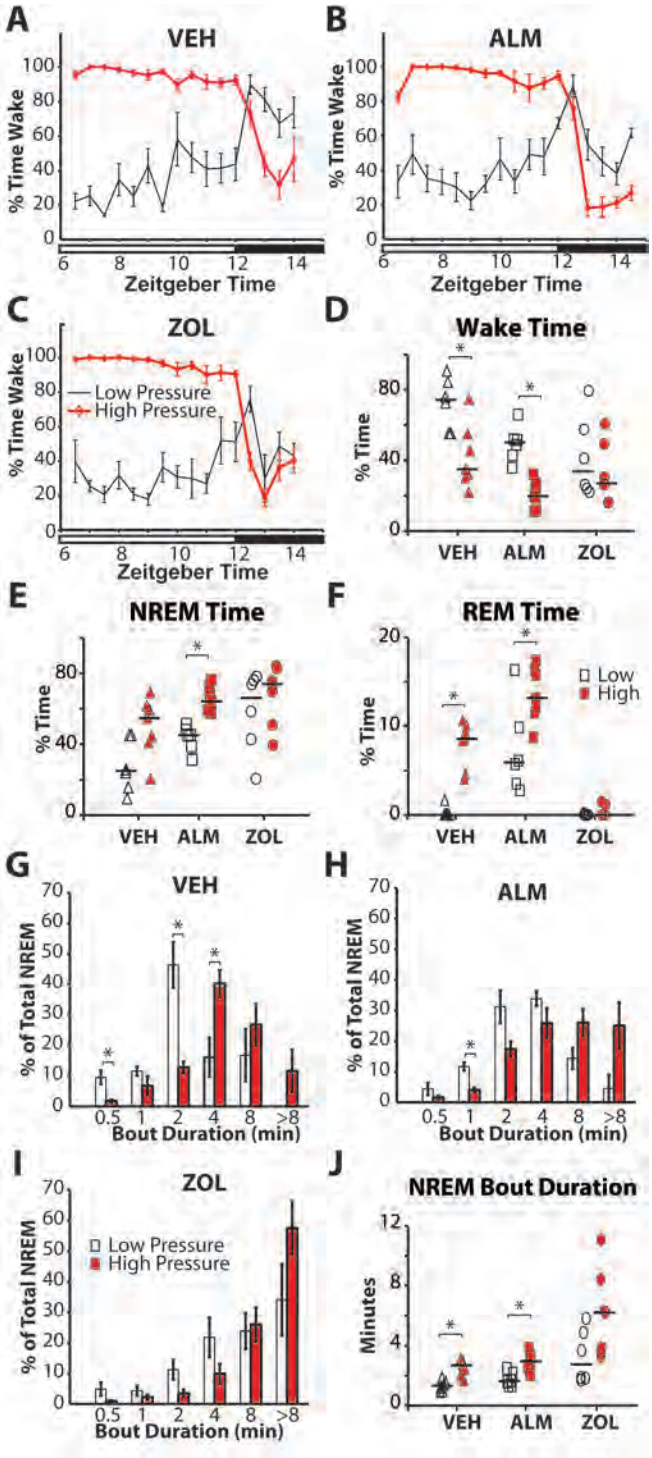
A
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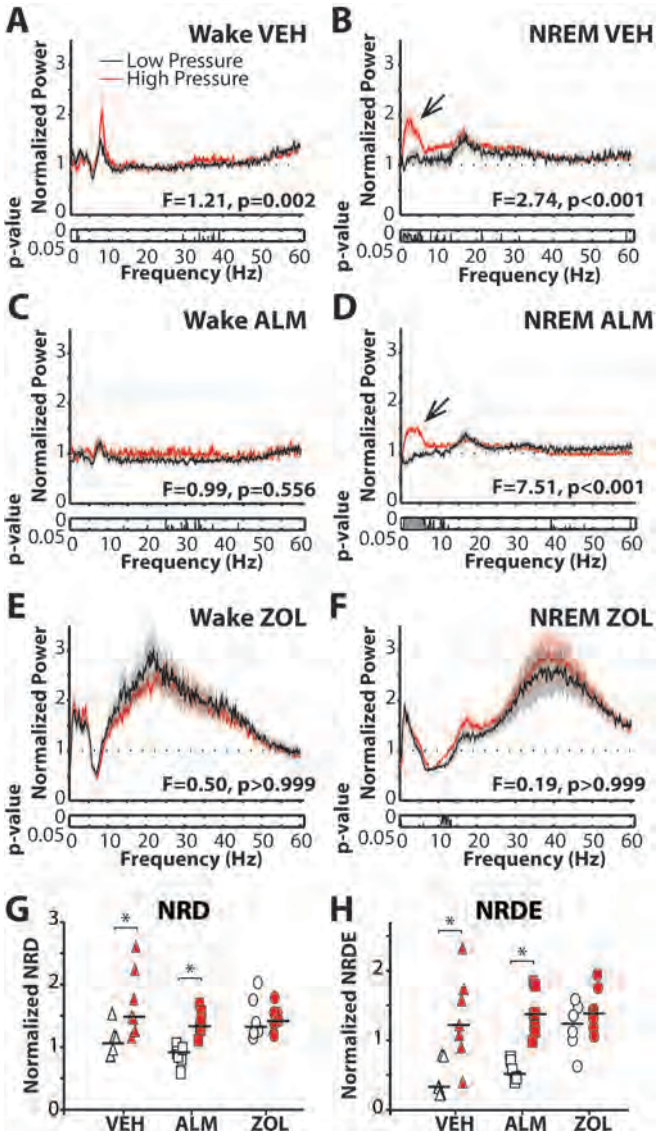
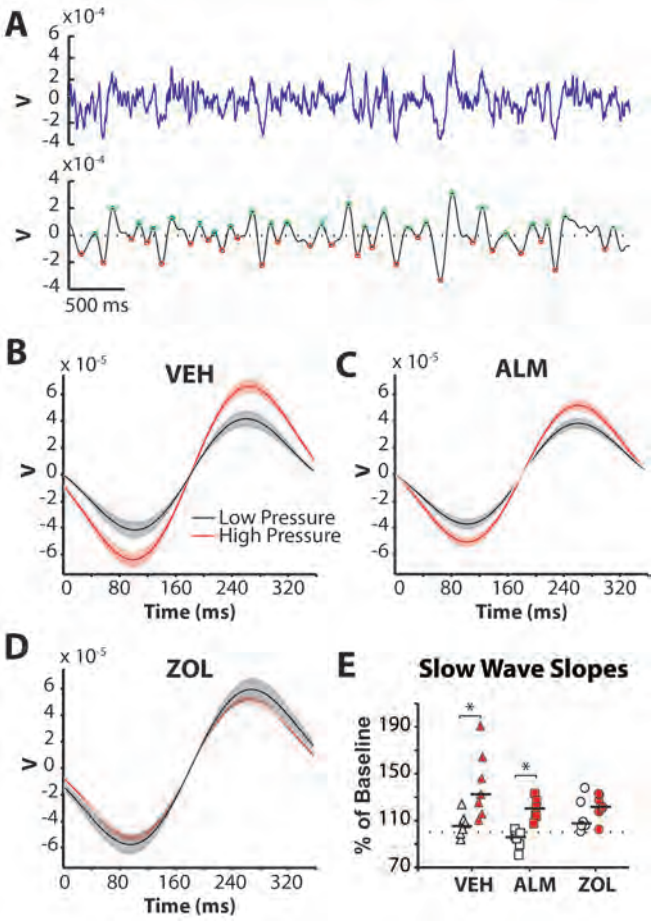
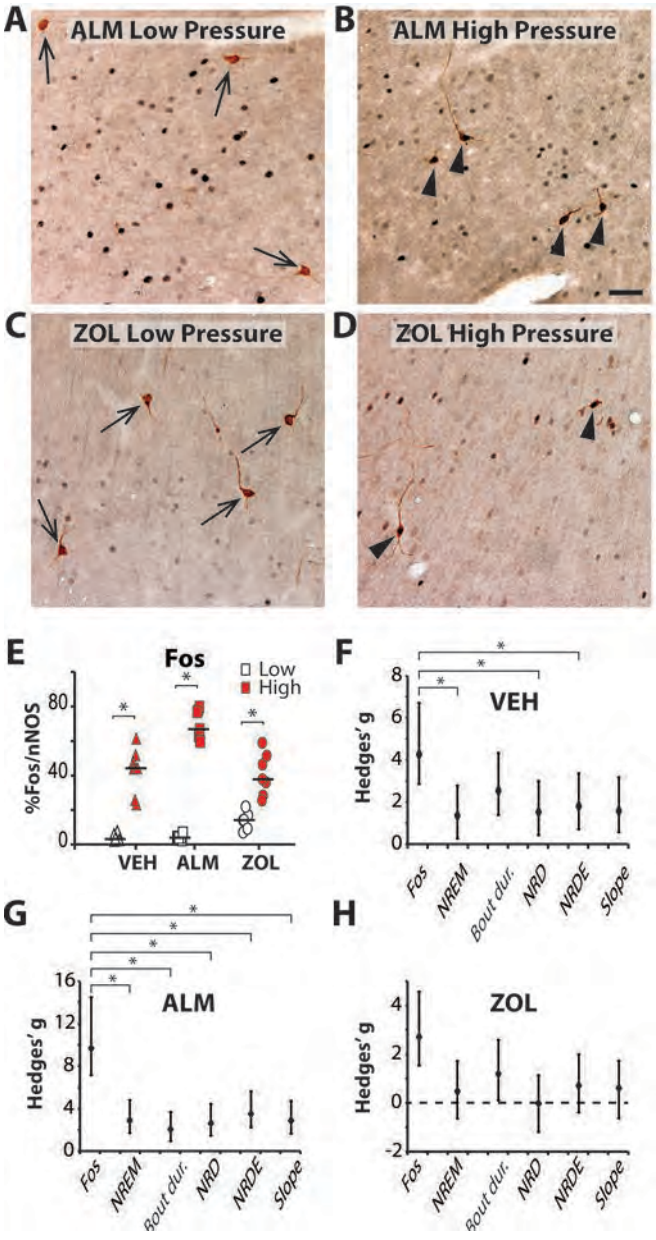

A
Wake VEH
Low Pressure
High Pressure
Normalized Power
F=1.21, p=0.002
p-value
Frequency (Hz)
B
NREM VEH
F=2.74, p<0.001
C
Wake ALM
F=0.99, p=0.556
D
NREM ALM
F=7.51, p<0.001
E
Wake ZOL
F=0.50, p>0.999
F
NREM ZOL
F=0.19, p>0.999
G
NRD
Normalized NRD
VEH
ALM
ZOL
H
NRDE
Normalized NRDE


A
x10-4
V
500 ms
B
VEH
x 10-5
Low Pressure
High Pressure
Time (ms)
C
ALM
D
ZOL
E
Slow Wave Slopes
% of Baseline
VEH
ALM
ZOL


A
ALM Low Pressure
B
ALM High Pressure
C
ZOL Low Pressure
D
ZOL High Pressure
E
Fos
Low
High
%Fos/nNOS
80
40
0
VEH
ALM
ZOL
F
VEH
Hedges' g
8
6
4
2
0
Fos
NREM
Bout dur.
NRD
NRDE
Slope
G
ALM
Hedges' g
16
12
8
4
0
Fos
NREM
Bout dur.
NRD
NRDE
Slope
H
ZOL
Hedges' g
4
2
0
-2
Fos
NREM
Bout dur.
NRD
NRDE
Slope